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PEYTON ROUS

HERBERT S. GASSER

RENÉ DUBOS

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WITH THIRTY-FOUR PLATES AND EIGHTY-TWO  
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# MORPHOLOGICAL AND OTHER CHARACTERISTICS OF THE AGENT OF FELINE PNEUMONITIS GROWN IN THE ALLANTOIC CAVITY OF THE CHICK EMBRYO

BY DOROTHY HAMRE, PH.D., HELEN RAKE, AND GEOFFREY RAKE, M.B.

(From the Division of Microbiology, The Squibb Institute for Medical Research,  
New Brunswick)

PLATES 1 AND 2

(Received for publication, March 8, 1947)

Examination of smears and sections of tissues stained with Giemsa or by Macchiavello's method led to the description of the developmental cycle of the Chlamydozoaceae (1), or the lymphogranuloma-psittacosis group of agents. However, because of their small size no details of structure could be seen before the development of the electron microscope. By the use of this instrument and the adoption of such methods of preparing material for examination as the shadow-casting technique of Williams and Wyckoff (2), and the Hillier and Baker (3) modification of replica preparation (Schaefer and Harker (4)), it is now possible to study in part the structure of the bodies of the agent. In a previous report (5) we presented electron micrographs of the agent of feline pneumonitis purified from yolk sac suspensions by differential centrifugation. During purification much of the agent was lost, and because of the complex nature of yolk, it was difficult to obtain clear preparations. These difficulties were largely overcome by using heavily infected allantoic fluid.

## *Growth of the Agent of Feline Pneumonitis in the Allantoic Cavity*

*Material and Methods.*—The passages were initiated by injecting 0.5 ml. of a 20 per cent yolk sac suspension into the allantoic cavity of 10 day old embryos. Eggs were harvested 4 to 5 days after inoculation and 0.5 ml. of either a 30 to 50 per cent suspension of the chorioallantoic membrane ground or shaken with beads in allantoic fluid from the same eggs, or allantoic fluid alone, was used for passage.

*Results.*—In early passages the number of elementary bodies seen in smears of the allantoic fluid was small. This is in agreement with the results of Francis and Gordon (6) who reported infection of 50 per cent of the eggs injected into the allantoic cavity with feline pneumonitis in the two passages they made. There were no deaths among our eggs until the 8th passage, and, in passages following that, the mortality varied from 0 to 30 per cent, but there was no definite trend toward higher mortality as passages increased, although the amount of agent in the allantoic fluid increased 100-fold from the 13th to the

50th passage (see below). The reason for the failure of the agent growing in the allantoic cavity to cause death consistently is not known; perhaps it is because the agent with its toxic factor does not invade the general circulation, or perhaps the lethal effect per infective unit of agent is decreased on prolonged cultivation in the allantoic sac.

At the 13th passage the amount of agent in the chorioallantoic membrane and in the allantoic fluid was determined by titration in the yolk sac, using 5 eggs for each tenfold dilution. The dose infectious for 50 per cent of the eggs ( $ID_{50}$ ), determined by the method of Reed and Muench (7), of the chorioallantoic membrane suspended in broth was  $10^{-4.7}$ , and of the allantoic fluid  $10^{-4.1}$ .

After 30 serial passages in the allantoic cavity, the amount of agent in smears of the allantoic fluid increased, and often "micro colonies" or clusters of elementary bodies were seen. These were circular in shape, sometimes larger than an oil immersion field and usually contained no recognizable fragments of cellular material.

When the amounts of agent in the chorioallantoic membrane and allantoic fluid were determined at the 50th passage (Table I), it was found that the  $ID_{50}$  of the allantoic fluid on the 5th day after inoculation was  $10^{-6.6}$  as compared with  $10^{-6.6}$  at the 13th passage. The titers of both the membrane and the fluid were highest on the 5th day following inoculation. On the 6th and 7th days the titer of the membrane changed little if at all, while that of the allantoic fluid fell significantly.

Multiplication of the agent in the allantoic cavity was slower than in the yolk sac where the amount of agent increased 4.5 logs between 24 and 48 hours after inoculation with  $10^{6.0}$  infectious doses (8). Also a much larger infective dose was required in the allantoic sac in order to obtain maximal titers of agent in 5 days. With inocula containing less than  $10^{4.0}$  infective doses, the agent could not be found in smears of allantoic fluid 6 days after infection.

Suspensions of chorioallantoic membranes in allantoic fluid from heavily infected eggs were rapidly fatal to mice when injected intravenously, indicating the presence of the toxic substance already described (9). This was confirmed by a toxin neutralization test using antiserum prepared in rabbits against the feline pneumonitis toxin in yolk sac suspensions (Table II).

Chorioallantoic membranes from 7 eggs were pooled, weighed, and shaken with combined allantoic and amniotic fluids from 3 of these eggs, so as to give 1 gm. of tissue to 3 ml. of total suspension. The membranes and fluids were those containing the most agent as shown by examination of smears stained by Macchiavello's method. The suspension was shaken with beads for 20 minutes, centrifuged at 2000 R.P.M. for 15 minutes, and then diluted with yolk and fluids from normal 6 to 8 day old embryonated eggs. In the titration 14 gm. Swiss mice were given 0.5 ml. intravenously of the dilutions of toxin shown in the table.

Serum from rabbit 7-5, bleeding 12/1, was obtained after 3 weeks of immunization with toxin prepared from yolk sacs heavily infected with the agent of feline pneumonitis. All injections were intravenous and the schedule described in the paper of Rake and Jones (10) for the preparation of antisera was followed.

This serum preparation had protected mice against two lethal doses of toxic factor derived from yolk sac at a dilution of 1/800 but not at 1/1000 when last tested 3 years before (9). For the present experiment serum dilutions were prepared in yolk and fluids from normal embryonated eggs so as to give, when mixed with equal parts of 1/3 suspension of toxin, a 1/6 dilution of toxin and twofold dilutions of serum from 1/200 to 1/1600. These mixtures were incubated at room temperature for 2 hours and then 0.5 ml. inoculated intravenously into 14 gm. Swiss mice. The results are shown in Table II.

TABLE I

*The Amount of Agent in the Chorioallantoic Membrane and in the Allantoic Fluid*

Time after inoculation	ID <sub>50</sub> of	
	Chorioallantoic membrane	Allantoic fluid
0 hour	10 <sup>-4.8</sup>	10 <sup>-7.2</sup>
1st day	10 <sup>-5.9</sup>	10 <sup>-6.4</sup>
2nd day	10 <sup>-7.9</sup>	10 <sup>-7.2</sup>
3rd day	10 <sup>-8.2</sup>	10 <sup>-7.2</sup>
4th day	10 <sup>-8.4</sup>	10 <sup>-7.5</sup>
5th day	10 <sup>-9.0</sup>	10 <sup>-8.5</sup>
6th day	10 <sup>-8.2</sup>	10 <sup>-7.2</sup>
7th day	10 <sup>-8.5</sup>	10 <sup>-6.5</sup>

TABLE II

*Toxin from Feline Pneumonitis Grown in the Allantoic Cavity and Its Neutralization by Specific Antitoxin*

Dilution of toxin	Dilution of antitoxin	Time of death of mouse No.				
		1	2	3	4	5
		hrs.	hrs.	hrs.	hrs.	hrs.
1/3	—	<18	<18	<18	<18	<18
1/6	—	<18	<18	<18	<18	<18
1/12	—	<18	22	25	25	<88
1/24	—	<42	<88	<88	<88	<112
1/6	1/200	<66	S	S	S	S
1/6	1/400	24	24	<42	S	S
1/6	1/800	<16	20	<42	<42	<42
1/6	1/1600	<16	<16	<16	<16	<20

Figures in bold-face type are deaths due to infection rather than toxin.

S indicates survival for 21 days.

*Method of Purification of the Agent for Photography.*—It was found that most of the agent was present in the sediment after centrifuging for 30 minutes at 3600 R.P.M. in an angle centrifuge.

The infected allantoic fluid, therefore, was purified by the following process. The first centrifugation, in an ordinary centrifuge at 800 R.P.M. for 10 minutes, removed most of the

cells. (Chilling of the eggs for 5 hours before harvesting the fluid prevented bleeding into the allantoic fluid.) The supernate was then spun at 3600 R.P.M. for 30 minutes in the angle centrifuge, and the sediment from this centrifugation was resuspended in saline or in distilled water and recentrifuged at the same speed for 30 minutes. The sediment was resuspended in a small volume of saline or distilled water, concentrating the agent 5 to 10 times. A small drop of this suspension was dried on the collodion-coated screen, and if saline had been used in the process of purification the screens were washed with distilled water. Some preparations were shadowed with gold (2) under vacuum of the order of 0.00003 mm. Hg in a device designed and built by Dr. H. Sidney Newcomer.

Replicas were made by pouring a solution of 1 per cent collodion in amyl acetate over a dried film of the suspension of agent on a clean glass slide. After carefully removing the collodion film by floating it off in water, screens were dropped onto the film, then removed with the adhering film, dried, and shadowed with gold, utilizing the device and method described in the preceding paragraph. Since the elementary bodies themselves are not exposed to high vacuum this technique does not contribute to the distortion of the agent.

The preparations were examined with an RCA electron microscope type EMU.

### *Morphological Characters*

Elementary bodies purified from allantoic fluid (Fig. 1) are similar in appearance to those purified from yolk sac (5). The irregularity of the surface caused by distortion of the body during drying, giving the body the appearance of a wrinkled half pea, is clearly shown in Fig. 2 of gold-shadowed replicas of the elementary bodies, and in Figs. 3 and 4 of the gold-shadowed bodies and the shapes of their shadows. Sometimes the bodies resemble hollow rubber balls with one side punched in (Fig. 5).

These pictures also show that at least two components make up the elementary body, a dense substance, usually centrally located, and a surrounding thinner substance which seems to represent a limiting membrane from which the central mass has shrunk away.

It is possible that these two components exist in the living state in the relation to one another shown in these photographs. However, it is probable that this apparent separation into two components may be due entirely to distortion of the bodies during drying.

Elementary bodies in chains, pairs, and clusters can be seen in Figs. 3, 5, 6, and 7. In clusters, the elementary bodies give the impression of possessing a sticky substance on their surfaces (Fig. 6).

The group of elementary bodies in Fig. 7 appears to be enmeshed in a matrix, composed of strands of material some of which are connected to the elementary bodies. The material in the background is somewhat similar in appearance to the ether-soluble antigen of *Rickettsia* shown in electron micrographs by Shepard and Wyckoff (11).

The average size of the gold-shadowed elementary bodies was calculated from measurements of 92 bodies. The mean diameter is 525 m $\mu$  with a standard deviation of  $\pm 84$  m $\mu$  and a standard error of  $\pm 5.5$  per cent. The size ranges from 303 m $\mu$  to 728 m $\mu$ , with 73 per cent of the measurements lying between

468 and 572  $m\mu$ . This variation in size of the elementary bodies can be seen in Fig. 4. Only a small number of measurements of unshadowed elementary bodies from allantoic fluid were made. From data on 15 elementary bodies, the mean diameter is 479  $m\mu$  with a standard deviation of  $\pm 44 m\mu$  and a standard error of  $\pm 4.88$  per cent. This agrees with previous measurements of elementary bodies from yolk sac suspensions, in which the mean diameter was 455  $m\mu$  (5).

When the height of the bodies was calculated from the length of the shadows and the angle at which the gold was cast, it varied from 175  $m\mu$  to 375  $m\mu$  for 23 measurements. This confirms the impression that the agent flattens out on drying and indicates that the figures given here for the mean diameter of the elementary bodies probably differ considerably from the true dimensions in the natural state. In this connection it might be pointed out that another related agent with very similar morphology both with the light and the electron (5) microscopes, *i.e.* that of lymphogranuloma venereum, has been found by direct measurement with a light microscope to be between 200 to 400  $m\mu$  in diameter (12, 13).

The impression gained from this study is that the elementary bodies of the agent of feline pneumonitis are either spherical or hemispherical bodies with a limiting membrane. Since they contain a large amount of water, an inference justified by the amount of distortion that occurs during drying, it is difficult to decide, from electron micrographs, what the actual shape of the bodies is.

#### SUMMARY

The agent of feline pneumonitis has been grown in the allantoic cavity for 50 serial passages. During this time the amount of agent in allantoic fluid increased about 100-fold. The titer of the agent in the allantoic fluid of the individual embryo reached a peak on the 5th day and then declined. Large inocula were required in order to obtain maximal titers. The toxic factor was present in suspensions of chorioallantoic membranes in allantoic fluid from heavily infected eggs, and could be neutralized by the specific antitoxin produced in rabbits by injection of toxin in yolk sac suspensions.

Electron micrographs of the agent of feline pneumonitis grown in the allantoic cavity show that the elementary body is composed of a dense centrally located substance surrounded by a thinner material, part or all of which is the limiting membrane. Separation of these two portions of the body may be due partially or entirely to distortion during drying. The wrinkled surface of the elementary bodies is evidence that such distortion does occur. The average diameter of gold-shadowed bodies is 525  $m\mu$ .

The authors are indebted to John J. Oskay for technical assistance.



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## EXPLANATION OF PLATES

## PLATE 1

FIG. 1. Elementary bodies not shadowed with gold.  $\times 14,160$ .

FIG. 2. Replicas of elementary bodies, gold-shadowed, 21.6 mg. of gold, angle  $22^\circ$ , 12 cm. distance.  $\times 14,160$ .

FIG. 3. Elementary bodies in chains, gold-shadowed, 23.5 mg. of gold, angle  $12^\circ$ , 10 cm. distance.  $\times 14,160$ .

FIG. 4. Elementary bodies gold-shadowed, 23.5 mg. of gold, angle  $12^\circ$ , 10 cm. distance.  $\times 14,160$ .

FIG. 5. A group of elementary bodies gold-shadowed, 23.5 mg. of gold, angle  $12^\circ$ , 10 cm. distance.  $\times 14,160$ .



PLATE 2

FIG. 6. Group of elementary bodies gold-shadowed, 21.7 mg. of gold, angle  $11^\circ$ , 10 cm. distance.  $\times 14,160$ .

FIG. 7. Elementary bodies enmeshed in a matrix, gold-shadowed, 23.5 mg. of gold, angle  $12^\circ$ , 10 cm. distance.  $\times 14,160$ .



(Hamre, Rake, and Rake: Agent of feline pneumonitis



# ABSORPTION FROM THE PULMONARY ALVEOLI

By CECIL K. DRINKER, M.D., AND ESTHER HARDENBERGH

(From the Department of Physiology, Harvard School of Public Health, Boston)

## PLATE 3

(Received for publication, March 25, 1947)

The ways in which molecules of different sizes, and finally visible particles, including bacteria, are absorbed from the lungs, present recurring problems to those concerned with many aspects of medicine and surgery. In this communication findings are recorded which have been gathered through the last four years with the advantage of new technical methods.

The routes of absorption from normal lung alveoli require passage through the epithelial lining of the alveoli and then the crossing of endothelium-lined blood capillaries into the blood or, if the second available route is to be followed, penetration of a lymphatic capillary through a delicate endothelial wall into the lymph stream. There is no doubt that bronchiolar absorption occurs, but both from the surface area available and the anatomical barriers imposed, it is of little importance compared with the absorption from the alveoli.

### *Experimental Technique*

In every case dogs have been the experimental animals and have been anesthetized with 35 mg. per kilo of pentobarbital sodium (nembutal) given intravenously and repeated whenever necessary. The route of absorption into the blood capillaries in the lungs needs no comment. Absorption through the lung lymphatics is not so familiar and the anatomical considerations governing this pathway for removal of foreign material require explanation, since the validity of the experiments which will be described depends upon knowledge of the variable arrangements of the lung lymphatics and ability to cope with these variations.

Lymph from the lungs enters the blood through the right lymphatic duct, only a very small part of the left upper lobe being drained by the thoracic duct. Although it is true that right duct lymph invariably represents drainage of the lungs, it is unfortunately the fact that connections between the thoracic duct and the right lymphatic duct are frequent. In the dog, Freeman (1) found that in 12 out of 25 animals there were connections between the two ducts. This, for man, if true, is of the highest importance when thoracic duct obstruction occurs, or when it becomes necessary to tie the thoracic duct near to entrance into the left subclavian vein. But for the investigator who wishes to collect lung lymph with no additions except an inevitable small increment from the heart, it becomes very trying to encounter a series of animals in which the right duct lymph is grossly similar to that of the thoracic duct, in that both contain chyle. But with patience and experience, it eventually develops that in about one experiment in three, the right lymphatic duct can be cannulated and will deliver no lymph save that from the heart and lungs. There are no reliable directions for finding the right lymphatic duct. The technique could be made to seem quite simple by directing the dissector to follow the right cervical lymphatic vessel down to its junction with the right duct. The difficulty is that this direct means of finding the right duct is reliably unreliable. The right duct in the dog usually joins the axillary vein just above the first rib. With sufficient fre-

quency to alleviate discouragement, one sees the right duct come clearly into view just above the first rib, and the entrance into the vein is plain. But lymphatics in all parts of the body have a versatile irregularity of position and connection, and consequently the right lymphatic duct may be found emptying into different veins in unexpected planes at the base of the neck. Fortunately, as has been described in some detail by Drinker (2), it is possible to ascertain whether the lymph being collected from the right duct is actually from the lungs. This is learned by the simple expedient of instilling 5 to 10 cc. of 0.5 or 1 per cent T-1824 in Ringer's solution into the lung alveoli. It will be found that this intense blue dye will usually color the right duct lymph in 20 to 30 minutes but will not be detectable in the thoracic duct lymph until much later. This latter development expresses the absorption of the dye by the blood passing through the lungs, and is merely an indication of the generalized distribution of the blue compound in the tissue fluid all over the body. What is of consequence is the fact that if T-1824 reaches the pulmonary alveoli, it is seen in a short time in lymph coming from the lungs. A further control of the situation provided by right duct cannulation can be gained by subcutaneous injection of a graphite or very dilute India ink solution into a hind foot. If the site of this injection is massaged and the foot moved passively, the black injection mass appears promptly in the thoracic duct lymph and is not evident in right duct lymph, unless there is connection between the two sides.

In summary, it is apparent that if the thoracic duct and right lymphatic duct are cannulated, and if diffusible foreign materials readily identified in low concentrations are instilled into the lung alveoli, the observer can ascertain whether absorption has been into the blood or into the lymph, and the time required for the beginning of removal as well as an approximation of the rate and probable success of alveolar clearance can also be obtained.

### EXPERIMENTS

The ease with which water and solutes up to about the dimensions of egg albumin are absorbed from the lungs is not adequately realized by physiologists and clinicians. The facts are that water leaves the pulmonary alveoli to pass into the blood capillaries with a degree of rapidity quite consonant with the profuse blood capillary area available for absorption in the lungs. Winternitz and Smith (3) instilled physiological salt solution into the trachea of an anesthetized dog and found prompt removal of large amounts of fluid. When phenol-sulfonphthalein was added to the salt solution it appeared in the urine at once. These experiments mean that there is no alveolar barrier to the passage of water nor of small molecules and that the lung capillaries carrying blood at a relatively low pressure are easily accessible and very effective as a means of absorption. It will enforce this statement to summarize a simple experiment:—

A dog anesthetized with nembutal was prepared for observation by cannulation of the thoracic and right lymphatic ducts. The obvious presence of chyle in the thoracic duct lymph and absence in right duct lymph indicated lack of communication between lung lymphatic and thoracic duct drainage. Ten cc. of a 1 per cent solution of T-1824 in physiological salt solution was instilled intratracheally, the dog being inclined head up at about a 30° angle during the instillation and for some minutes afterwards. Within 20 minutes the right duct lymph became blue and in a short time blood specimens showed a faint blue tinge in the plasma. This meant that in a dog with no possibility of lymphatic delivery of dye into the blood, molecules of this

dye passed through the alveolar epithelium to reach the alveolar blood capillaries and then entered the blood. At the same time dye molecules reached the lung lymph on the way to the right duct and delivery into the right axillary vein. Cannulation of the right duct disclosed this path of removal from the lungs, but though the concentration of dye in the lymph was very high, so that the fluid collected was an opaque deep blue, one could not escape the fact that simultaneously with lymph absorption of dye molecules there had been a steady and efficient absorption directly into the blood. Had there been no interference with lymph delivery to the blood through the normal route, blood concentrations of dye might have become higher, but this obvious point is not the true concern of the experiment.

It is the fact that intraalveolar molecules, even of fairly large size as represented by the dye T-1824, enter the blood capillaries quickly and the main route for lung clearance of such foreign instillations is the blood. If lymphatic connections are normal, that is, unbroken by the cannulations possible to the experimenter, additional increments of dye absorbed by the lung lymphatics will reach the blood through the right duct and to a very minor degree through the thoracic duct.

These facts are of more than experimental significance. For example, penicillin by inhalation was advocated early in the history of the drug for direct medication of bronchiectatic cavities and suppurative conditions in the lungs. It is doubtful how far inhaled mists containing penicillin reach suppurative foci which it is hoped they will affect favorably. At the same time there cannot be any question that the intrapulmonary absorption of inhaled penicillin is extremely effective and one can rely on high blood titres of penicillin administered by inhalation.

It is now known (4) that T-1824 forms some sort of combination, chiefly with albumin, and in this way the molecule of blue dye is effectively increased in size and diffuses through living membranes very slowly. If T-1824 is vaporized in watery solution and a dog breathes the blue mist, which is wholly non-irritating, the blood plasma becomes strikingly blue in a short time. There is absorption by the lung lymphatics, as cannulation of the right lymphatic duct shows, but it is insignificant in amount compared with the ready entrance into the blood.

In contrast to the experiments upon ready absorption of T-1824 in water, a number of experiments have been done in which the dye, in 0.5 to 1 per cent concentration, has been dissolved in Ringer's solution and then various proteins added, giving in each case a concentration of 2.0 to 5.0 per cent of protein. At the start, fresh heparinized dog plasma was used; later, highly purified serum albumin; and finally, egg albumin. In all these cases the dye combines with protein, and on dialyzing the blue solution against Ringer's solution many hours are required before the salt solution shows recognizable traces of blue color. In addition to these proteins, experiments were also made with hemoglobin solutions. Hemoglobin does not combine with T-1824. It was our hope that



if absorption into the lymph occurred, it would be evident through red color in the right duct lymph. This did not happen to a recognizable degree.

Typical experiments are as follows:—

1. *Blood Plasma Plus T-1824.*—

Dog weight, 11.8 kg.

9:15 a.m. 9 cc. of 5 per cent nembutal, intravenously.

10:05. Intratracheal injection of 6.0 cc. of a solution which contained 0.5 per cent T-1824 plus heparinized dog plasma making an eventual concentration of 4.4 per cent protein.

10:15. 5 cc. same solution intratracheally. During these instillations the board was elevated for 15 minutes so as to give a 30° angle, head up position.

10:35. 200 cc. Ringer's solution intravenously—slowly.

11:25. Thoracic duct cannulated. Lymph flow rapid.

11:30. Right lymphatic duct cannulated. Lymph flow excellent.

11:42. Blood pressure record from left femoral artery.

11:48. Second blood pressure record.

11:49. Artificial respiration given through tracheal cannula by means of a pump supplying air under positive pressure for inspiration, expiration being normal, that is, without suction. The pump used can be set for rate and minute volume of delivery where the animal ceases all efforts to breathe in its natural manner.

12:10 p.m. 5 cc. of blood from left external jugular vein. Centrifuged at once. No blue color.

12:56. 1 cc., 5 per cent nembutal, intravenously.

1:13. 1 cc., 5 per cent nembutal, intravenously.

1:32. Third blood pressure record.

1:56. 1 cc., 5 per cent nembutal, intravenously.

2:10. Fourth blood pressure record.

2:11. Dog bled to death from femoral artery. Blood specimen centrifuged. No blue color.

*Autopsy.*—The largest part of the blue solution given intratracheally entered the lower right lobe which was intensely blue. There was a small degree of blue color posteriorly in all the other lobes on both sides.

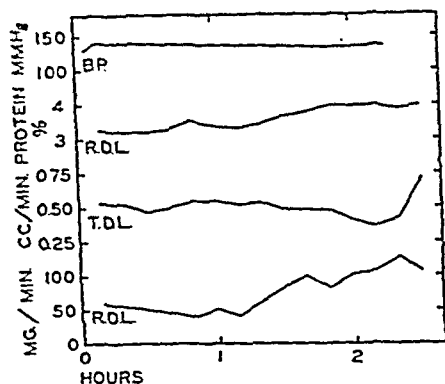
The right lymphatic duct and the vessels combining to form it showed no blue discoloration. The right tracheobronchial lymph node was very faintly blue, indicating slight absorption but not enough to pass through the node and reach the draining trunks leading eventually to the right lymphatic duct, the lymph from which never showed blue color.

*Comment.*—In this animal, observed for 4 hours after intratracheal instillation of a T-1824 and blood plasma solution, there was no delivery of the solution to the blood nor to the right lymph duct and thoracic duct. Experience has shown that had the solution been protein-free, right duct lymph would have become intensely blue in about 20 to 30 minutes and the blood appreciably so within the first hour.

The animal remained in excellent condition throughout the entire experiment. Positive pressure artificial respiration was applied, not because the condition of the dog required it, but because the squeezing effect on the lungs of the positive inspiratory blast of air greatly intensifies lymph drainage from the lungs

and assures the appearance of the blue solution in the right duct lymph, since it is forced out into the interstitial tissue of the lungs and then into capillary lymphatics. When such an injection is made directly into the lung parenchyma, though part of the blue solution is intraalveolar, the protection against absorption offered by the normal alveolar epithelium is lost to a fair degree and even if the solution does contain protein, lymphatic entrance is readily accomplished. This fact must be clear enough since one of the functions, indeed, possibly the most important function under normal conditions, of the capillary lymphatics is to remove excess protein which has escaped from the blood capillaries.

In Text-fig. 1, the details of blood pressure, protein content and flow of right duct lymph, and flow of thoracic duct lymph are given for the experiment in which plasma plus T-1824 was given intratracheally.



TEXT-FIG. 1. The details of Experiment 1 in which T-1824 in a Ringer-plasma solution was given intratracheally to a dog. Curves from top to bottom: *B.P.*, blood pressure in millimeters of mercury; *R.D.L.*, right duct lymph protein in grams per cent; *T.D.L.*, thoracic duct lymph in cubic centimeters per minute; *R.D.L.*, right duct lymph in milligrams per minute. Ordinates, as designated. Abscissae, time in hours.

## 2. Serum Albumin.—

In this experiment the animal was anesthetized with nembutal, the thoracic and right lymphatic ducts cannulated, and 11 cc. of a 4 per cent solution of purified bovine serum albumin, obtained from Dr. Charles A. Janeway, was instilled intratracheally.

The serum albumin employed was a crystallized bovine albumin prepared by Dr. Walter L. Hughes, from the Department of Physical Chemistry at the Harvard Medical School, and in use by Dr. Janeway for immunological experiments. It was a 24 per cent solution in isotonic saline with a pH of about 6.8. It contained merthiolate, 1 to 15,000, as a preservative. Dr. Janeway considered that the preparation contained about 0.1 per cent of globulin, being, thus, 99.9 per cent pure albumin. This stock solution was diluted to 4.0 per cent prior to use in the lung absorption experiments. No T-1824 was added.

Dr. William H. Batchelor, a student in Dr. Janeway's laboratory, determined the bovine

albumin content of blood and lymph specimens as follows: The crystallized bovine albumin content of the specimen received was determined by precipitation, using rabbit antiserum in the range of antibody excess. The nitrogen content of the washed precipitate was determined by a micro-Kjeldahl technique. Using a calibration curve, prepared for the particular antiserum, these nitrogen values (actually the equivalent of the nitrogen values in terms of the amount of  $N/70$  HCl needed to neutralize the ammonia) were compared with those gained by using known amounts of protein. Dog serum proteins do not yield precipitates with the antiserum. The error is probably within 20 per cent.

Artificial respiration was employed intermittently in order to accelerate the flow of lung lymph.

Specimens of lymph from the right duct and the thoracic duct were taken at approximately hourly intervals for 4 hours following intratracheal administration of the albumin solution. Blood specimens were secured before, during, and at the close of the 4 hour period of observation.

Five samples of thoracic duct lymph were all negative for albumin as were the blood samples.

The results relative to the right duct lymph were as shown in Table I.

TABLE I

*The Detection in Right Duct Lymph of Crystalline Bovine Albumin Dissolved in Saline and Introduced Intratracheally in the Dog*

Time	No. of specimen	Albumin (+ or -)	Amount of specimen	Remarks
<i>p.m.</i>		<i>mg. per cent</i>	<i>gm.</i>	
12:15-1:25 1:40	1		1.3	Instillation of albumin solution
1:25-3:00	2	Trace	1.2	
3:00-4:30	3	5.75	1.4	
4:30-5:40	4	25.75	1.3	

*Comment.*—In this case it is evident that a small degree of transfer of intra-alveolar albumin to the right lymphatic duct began shortly after instillation and increased over the next few hours but never reached high values. It must be remembered that right duct lymph drainage was forced by artificial respiration and would have been less under normal conditions of breathing. Also, it is significant that even at the close of this experiment the blood did not contain a detectable amount of albumin.

### 3. Egg Albumin.—

In this experiment a dog was given nembutal intravenously and the thoracic duct and right lymphatic ducts were cannulated. A cannula was inserted in the trachea to permit artificial respiration. Purified egg albumin, supplied by Dr. Gertrude E. Perlmann from the Massachusetts General Hospital, was made from fresh eggs, the egg white being recrystallized three times and stored at 3°C. under a saturated solution of ammonium sulfate. Before use, the preparation was dissolved in distilled water and dialyzed for 24 hours against running

distilled water, and then against smaller volumes of distilled water until ammonium sulfate-free. The dialysis was carried out at 3°C. The eventual solution was found to contain 6.9 per cent egg albumin. This solution was then diluted to 4 per cent albumin and 1 per cent T-1824 in 0.9 per cent saline. It was in turn dialyzed against 0.9 per cent salt solution and no release of T-1824 occurred. One can, therefore, conclude that in the case of egg albumin, there is a combination of T-1824 with the protein, and the blue dye serves as an indicator of the movement of the egg albumin molecule. In this experiment 11 cc. of 4 per cent albumin plus 1 per cent T-1824 were given intratracheally at 12:40 p.m., the dog being inclined head up during the instillation and for a short time thereafter. The results in right duct lymph are summarized in Table II.

*Comment.*—In the case of purified egg albumin there is evidence that slight traces of the protein did pass through the alveolar walls and reach the right duct drainage route, but absorption was just recognizable and no blue color was detected in thoracic duct lymph nor in blood plasma.

TABLE II

*Results Obtained in Right Duct Lymph When a Solution of Purified Egg Albumin Plus T-1824 Was Instilled Intratracheally*

Time	Lymph	Protein	Remarks
<i>p.m.</i>	<i>gm.</i>	<i>per cent</i>	
12:25–12:40	0.54	3.12	
12:40– 1:40	1.3	3.03	Intratracheal instillation egg albumin plus T-1824 at 12:40
1:40– 2:40	1.5	2.72	
2:40– 3:40	1.3	3.01	Questionable grey-blue color in lymph
3:40– 4:40	1.6	3.00	Definitely blue lymph
4:40– 4:58	0.73	3.03	
4:58– 5:05	0.31		All specimens very slight bluish tint
5:05– 5:18	0.22		
5:18– 5:25	0.20	3.03	
5:19			Blood specimen shows no blue color

#### 4. Hemoglobin.—

In several experiments crystalline hemoglobin in Ringer's solution and crude hemoglobin from washed red cells were given intratracheally. If absorption occurred during 4 hours, it was so slight as to be masked by the usual slightly yellow color of the right duct lymph.

#### 5. Visible Particles.—

Lymph from the right duct of the dog does not contain visible foreign particles even if the lung lymphatics and lymph nodes are plentifully loaded with black dust of microscopic dimensions. To determine the absorption of particles from the alveoli of the normal dog, it is necessary to examine sections of the

seen in Fig. 1, showed spheres attached to typical lung phagocytes, but in the space of 4 hours none of these cells carrying foreign particles had made visible progress along the conventional line of lymph drainage, and no spheres were seen in lymph nodes.

This result, in which a distinctive particle was employed, does not permit wide generalization. Because glass spheres averaging 4 micra in diameter were not moved in 4 hours from the alveoli, even to lymph nodes at the root of the lung, does not mean that other foreign particles might not make a quicker entrance in the lymph stream and a more rapid movement to lymph nodes. There can be no doubt that different physical and chemical characteristics of foreign particles in the alveoli influence the rapidity of removal by the lymphatic route.

In order to see whether great increase in lymph flow might cause movement of the glass spheres, dogs were given alpha-naphthyl thiourea (ANTU) which causes a huge outpouring of lung lymph. In a typical experiment (Text-fig. 2) the glass bead suspension was given intratracheally 3 hours and 5 minutes after intravenous ANTU injection had begun to increase lymph flow. This animal died 1 hour and 27 minutes later. At autopsy, the pleural effusion and lung edema which are caused by ANTU were found; but, on microscopic examination of lung tissue and lymph nodes, presence of the glass spheres in the lymphatic system could not be detected. Text-fig. 2 shows the details of this experiment.

#### DISCUSSION

Drinker, Warren, and MacLanahan (5) investigated the absorption of protein solutions placed in the alveoli of anesthetized dogs. Contrary to their expectations, they found that detectable traces of protein appeared in the blood some time before they were found in the thoracic duct lymph. The technique of preparing the animals for these experiments was inadequate in view of what has been learned relative to lymphatic drainage of the lungs in the last few years.

In 1937, it was thought that if the right lymphatic duct was tied where it makes venous entrance and the thoracic duct cannulated, all material absorbed from the lungs would be found in the thoracic duct lymph or in the blood. Although the right duct and the thoracic duct are often connected, the anastomosing vessels are usually very small and of little significance unless the thoracic duct is obstructed, when the increased lymph pressure forces dilatation, and eventually they become large enough to carry all of the thoracic duct lymph to the right subclavian vein. Under normal conditions of breathing the flow of lymph from the lungs is small, and if the right duct, into which the lung lymph flows, is obstructed, there is a large enough distribution of lymphatics in the lungs so that there is lymph accumulation in the lungs and practically no tendency to force the fluid into the thoracic duct through the small connecting vessels which may be present. One cannot expect in a brief experiment to obtain material absorbed from the lung alveoli in thoracic duct lymph. It is

necessary to cannulate the right lymphatic duct in order to accomplish this, just as has been done in the experiments described in this paper. Drinker, Warren, and MacLanahan (5) used qualitative immunologic methods for detecting horse serum, crystallized hemoglobin, and crystallized egg albumin after instilling large amounts (20 to 50 cc.) of solutions of these proteins into the alveoli by the tracheal route. Frequently the experiments lasted 24 hours, and it was found that after several hours all these proteins could be detected in the blood and later in thoracic duct lymph to which they had undoubtedly been delivered by the blood. These results, though inadequate for uncovering all the possible methods of absorption from the alveoli, do, however, indicate that so far as removal by the blood capillaries is concerned, exceedingly small amounts of foreign protein pass through the alveolar epithelium and then the blood capillary endothelium.

In the experiments reported in the present paper it is apparent that in 4 hours' time small protein molecules, notably serum albumin and egg albumin, do find their way into the lung lymphatics, though by using artificial respiration with a positive pressure delivery of air for inspiration, advantage has been taken of the measure found most effective in promoting absorption from the alveoli into lymphatics and the flow of lung lymph. The conclusion to be drawn is that unchanged transudates and exudates resulting from lung injury are removed from the lung alveoli in minute amounts. To clear the lungs of plasma proteins requires breakdown of molecules by enzymatic action until products are formed which are small enough to diffuse readily into the blood. The prolific supply of lymphatics in the lungs is apparently for the purpose of slowly moving wholly insoluble substances into the lymph stream, with the usual result of imprisoning them in lymph nodes prior to a possible entrance into the blood and general distribution throughout the body. The chief barrier to absorption is apparently the alveolar epithelium, since, when proteins are injected directly into the lung tissue and so diffuse widely in the alveolar walls, delivery of these substances by the right lymphatic duct is very prompt.

The results reported in this paper and accumulated over a number of years, accord with and extend those obtained by Cameron and Courtice (6) and by Courtice and Phipps (7).

#### SUMMARY

Experiments upon dogs anesthetized with nembutal and lasting 4 hours, in which the right lymphatic duct and thoracic duct have been cannulated and collection of lung lymph and blood specimens was accomplished after intra-tracheal instillation of dog plasma, purified bovine serum albumin, crystallized egg albumin, and hemoglobin, have shown that the absorption of such molecules is slight. Experiments in which pyrex glass spheres averaging 4 micra in

diameter were instilled failed to disclose entrance of these distinctive foreign particles into the lymph stream, though the fact that lung phagocytes were often found containing the particles or covered with them, indicated that eventually these particles would be found in lung lymphatics and in lymph nodes. The protection against absorption from the lung alveoli is in the main due to intact alveolar epithelium through which molecules of the dimensions of the proteins commonly entering the alveoli, as a result of trauma or disease, pass very slowly and are found in small traces in lung lymph and even to a less degree in blood.

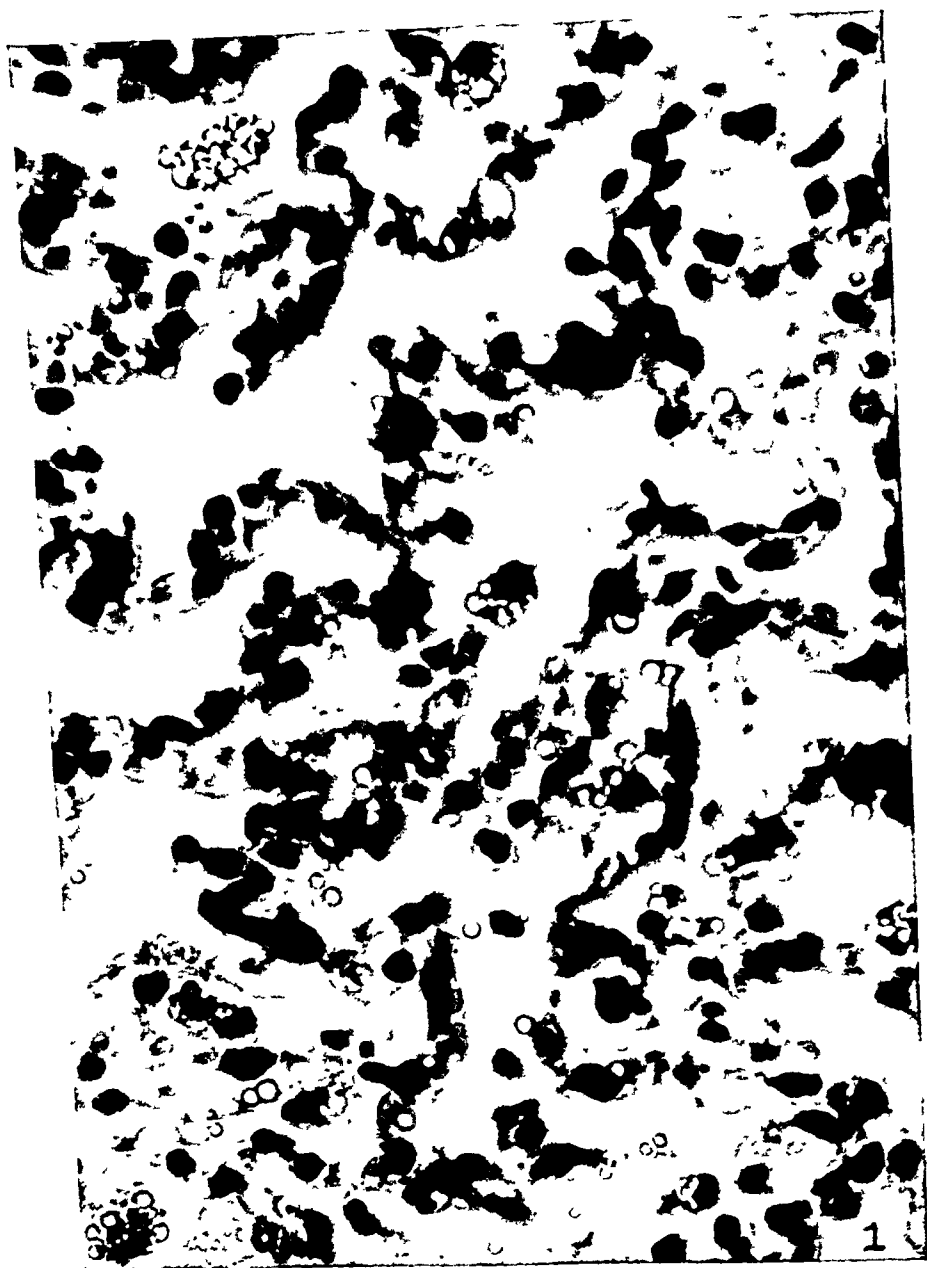
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## EXPLANATION OF PLATE 3

FIG. 1. Photomicrograph of the lung of a normal dog into which glass spheres, 4 micra in average diameter, have been instilled intratracheally. Many spheres have been collected by phagocytes, many are free in the alveoli, and here and there one sees possible but not certain entrance of a sphere into the alveolar wall.  $\times 550$ .





(Drinker and Hardenbergh Absorption from pulmonary alveoli)



# STUDIES ON SPREADING FACTORS

## II. THE EFFECT OF SERUM UPON HYALURONIDASE SPREADING ACTIVITY\*

By OSCAR HECHTER, Ph.D., AND ELLA L. SCULLY

(From the Worcester Foundation for Experimental Biology, Shrewsbury, and the Department of Physiology, Tufts Medical School, Boston)

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### INTRODUCTION

The fact that normal serum from non-immunized animals contains a factor which inhibits certain activities of hyaluronidase now seems well established (1-3). Although the biological significance of this antihyaluronidase factor is obscure, it has been recently suggested that the serum factor is the fundamental body defense mechanism against invasive processes catalyzed by hyaluronidase (3). This view, however, is based on an extrapolation of *in vitro* findings to invasion in skin. It should be noted that there is little or no information concerning the effect of serum upon hyaluronidase spreading activity in skin and that the principal evidence for the existence of the serum antihyaluronidase factor is derived from *in vitro* studies on decapsulation of streptococci (1), removal of follicle cells from mammalian ova (2), and viscometric studies using hyaluronic acid preparations (3).

Since the spreading effect of hyaluronidase in skin is the activity of hyaluronidase which has been implicated in bacterial, and other, invasive processes, the need for an understanding of the effect of the serum factor upon hyaluronidase action in skin is clear, particularly since it is known that the activity of hyaluronidase in skin is markedly influenced by non-enzymatic factors (4). To date, the only information available concerning the effect of the serum factor upon hyaluronidase spreading activity is: (a) an isolated statement by Humphrey (5) that serum inhibits the spreading activity of testicular hyaluronidase, with no details of this work presented, and (b) the illustration of a similar inhibitory effect of rabbit serum in spreading of vaccine virus by Duran-Reynals (6) although this observation was not mentioned in the text of the paper.

Accordingly, studies were undertaken to determine the effect of serum upon hyaluronidase spreading activity. In this paper the results of these studies will be described and the significance of the serum antihyaluronidase factor as a body defense mechanism against invasive processes potentiated by hyaluronidase will be discussed.

\* Aided by a grant from G. D. Searle and Company.

### Methods

The plan of these experiments has been to study the reaction between serum and hyaluronidase, under both *in vitro* and *in vivo* conditions. Hyaluronidase was measured using an assay method based on spreading activity which will be described later in this section of the paper. It should be emphasized immediately that although the spreading method of hyaluronidase assay is highly sensitive, the quantitative evaluation of hyaluronidase activity with this method is much less satisfactory than with simple *in vitro* tests using the viscosimeter (3), mucin clot prevention (7), or turbidimetric (8) methods of assay. Consequently, for a study of the *in vitro* reaction between serum and hyaluronidase, one of the latter procedures would be the method of choice. In this study, however, we are interested in the reaction between serum and hyaluronidase under environmental conditions in skin, and thus the spreading method is most informative for this purpose.

Hyaluronidase spreading activity was measured using hemoglobin or methemoglobin as an indicator in shaved rabbit abdominal skin. The measurement of areas of spread, preparation of indicator solutions, and of the solutions for intradermal injection have been described in a previous paper (4). The hyaluronidase preparation used in these studies was a partially purified bovine testis preparation (Schering). Rabbit and pig serum was used in these studies. We have found no significant differences in antihyaluronidase activity between normal serum and the serum derived from defibrinated blood. The samples of the pig sera used contained high *in vitro* antihyaluronidase activity as determined by the viscometric technic of Haas (3). Thus, 0.1, 0.2, and 0.3 cc. of these sera, incubated for 10 minutes with 50  $\mu$ g. of hyaluronidase in a total volume of 3 cc. inhibited hyaluronidase viscosity—reducing activity an average of 75, 85, and 90 per cent respectively. Rabbit serum, while not tested simultaneously as regards its inhibitory activity in the spreading reaction and in the viscosimeter, contains an approximately equivalent amount of antihyaluronidase activity as measured in the latter test (9).

Evaluation of the effect of *in vitro* incubation of serum with hyaluronidase was obtained by assay of hyaluronidase spreading activity using the following procedure: To eliminate the factor of animal variation, all tests of spreading activity in a given experiment were made in the same animal. Initially, varying concentrations of enzyme in a constant volume and indicator concentration were injected into duplicate sites and the area of spread 1, 2, 5, and 10 minutes after injection determined. After the dosage-response curves of hyaluronidase had been obtained, concentrations of enzymes were selected for *in vitro* incubation at 25°C. with serum. At varying intervals after the initiation of the *in vitro* reaction, a sample was removed and the spreading activity was determined in the same animal. To check the results obtained in the single animal, all tests for results were repeated at least three times with three different rabbits.

The quantitative aspects of the method require some mention. Twofold differences in enzyme concentration can be detected *only on the linear portion of the sigmoid dosage-response curve*. This method therefore has quantitative significance only in certain dosage ranges, and at best is not capable of detecting slight differences in hyaluronidase concentration.

In order to assess the *in vivo* inhibitory activity of serum uncomplicated by factors involving the rate of diffusion of antihyaluronidase through the capillary wall, etc., the effect of incubating serum with hyaluronidase in skin was studied. In these experiments, hyaluronidase and serum were mixed and immediately thereafter injected intradermally in a small volume (0.1 cc.) of fluid. After varying intervals of time, the residual spreading activity of the bleb was tested by reinjecting the initial site with 1.0 cc. of indicator solution. The basis for this assay depends on the fact that the final area of spread of intradermally injected hyaluronidase is directly related to the volume of injection; and that administered hyaluronidase in the absence of the pressure of injection, diffuses only slowly through skin (4). As a consequence of this, a portion of the injected hyaluronidase may be expected to remain in the area of injection. This hyaluronidase is available for further spreading activity if a second large injection

of fluid without enzyme is made into the area initially treated with enzyme. If, serum is introduced with the spreading enzyme and inactivates the hyaluronidase in the skin, the rate of spread of the second injection of a large volume of fluid should be correspondingly decreased. In these experiments, on *in vivo* incubation of serum and hyaluronidase the large areas of spread following the administration of the second injection limited the number of injections

TABLE I

*The Effect of in Vitro Incubation of Rabbit Serum with Varying Concentrations of Hyaluronidase (Serum Concentration Constant)\**

Final concentration before injection		Time after injection, min.‡						Average I
		1		2		3		
Hyaluronidase	Serum	A§	I§	A	I	A	I	
μg./cc.	cc./cc.	cm. <sup>2</sup>	per cent	cm. <sup>2</sup>	per cent	cm. <sup>2</sup>	per cent	per cent
0	0	1.70	—	2.04	—	2.36	—	—
0	0.3	1.74	—	1.94	—	2.42	—	—
0.1	0	1.70	—	2.08	—	2.70	—	—
1.0	0	1.97	—	2.53	—	3.28	—	—
2.0	0	2.80	—	3.60	—	4.34	—	—
	0.3	1.84	85	2.48	50	3.08	65	67
5.0	0	3.65	—	3.87	—	4.53	—	—
	0.3	1.69	98	2.76	78	3.46	78	84
10.0	0	3.72	—	4.47	—	5.01	—	—
	0.3	2.12	89	2.51	90	3.46	90	90
100.0	0	3.79	—	4.30	—	4.85	—	—
	0.3	4.02	0	4.50	0	4.78	0	0

\* Incubated at 25°C. for 10 minutes.

† All solutions were injected intradermally in a volume of 0.20 cc.

‡ A, area of spread in cm.<sup>2</sup>

|| I, Inhibition produced by *in vitro* incubation, in per cent, as evaluated from the dosage-response curves.

that could be made into the abdominal skin; thus it was not possible to make duplicate injections as in the case of those experiments involving *in vitro* incubation of serum with hyaluronidase. As in the previous case, the results of the single experiment were checked by repeating the test in three different rabbits.

#### EXPERIMENTAL

*Effect of in Vitro Incubation of Serum with Hyaluronidase.*—The effect of incubating varying concentrations of hyaluronidase with a constant amount of

rabbit serum was studied in seven experiments (four using pig serum and three with rabbit serum). Essentially similar results were obtained with both types of serum and the results of a single experiment with rabbit serum are shown in Table I.

In this experiment, 0.3 cc. of rabbit serum was incubated with 0.2 cc. of solutions containing 10, 25, 50, or 500  $\mu$ g. hyaluronidase per cc. for 10 minutes at 25°C.; 0.5 cc. of the hemoglobin indicator was then added so that the final concentrations were equivalent to 2, 5, 10, and 100  $\mu$ g. per cc. of hyaluronidase. A 0.2 cc. sample of the incubation mixture was then injected intradermally and the rate of spreading determined. The degree of inhibition produced by serum was determined by comparing the spreading activity of the incubated samples with the dosage-response curve of hyaluronidase alone.

It will be seen from Table I that the spreading activity of hyaluronidase is inhibited by 10 minutes *in vitro* incubation with serum. Thus, 2, 5, and 10  $\mu$ g. hyaluronidase incubated with 0.3 cc. serum in a volume of 0.5 cc. is inactivated approximately 67, 84, and 90 per cent respectively as regards spreading activity. The finding that 100  $\mu$ g. of hyaluronidase is not significantly inhibited under these conditions, does not mean that some degree of hyaluronidase inactivation did not occur. It should be pointed out that in order to demonstrate an inhibition with this concentration of hyaluronidase, almost 100 per cent of the spreading activity would have had to be removed.

In the next experiments, the effect of varying the serum concentration, using a constant amount of hyaluronidase, upon inhibition of spreading activity was studied. The results of four experiments of this type (three with rabbit serum, one with pig serum) were essentially similar, and the results of a single representative experiment are shown in Table II.

After determination of the dosage-response curve of hyaluronidase in a rabbit (*cf.* Table II) using a constant injection volume of 0.2 cc. a concentration of hyaluronidase was selected for incubation with varying amounts of rabbit serum so that the final concentration of hyaluronidase was 2.5  $\mu$ g. per cc. Then, 0.25 cc. of a 10  $\mu$ g per cc. solution of hyaluronidase was mixed with 0.05, 0.125, or 0.25 cc. of rabbit serum, saline was added to make a total volume of 0.5 cc., and the mixture was incubated for varying intervals of time at 25°C. At the completion of the incubation period, 0.5 cc. of indicator solution was added, the solution mixed, and spreading activity measured by intradermal injection of 0.2 cc. of the mixture. The degree of inhibition produced by serum was obtained by comparing the response with the previously determined dosage-response curve.

It will be seen from Table II that the inhibitory action of serum incubated *in vitro* with hyaluronidase is dependent both upon the serum concentration and the period of incubation. Thus, for a 10 minute incubation period, the undiluted serum (present in a final concentration of 0.25 cc. serum per cc. of the injected mixture) inhibited 2.5  $\mu$ g. of hyaluronidase to the extent of about 70 per cent; serum diluted 1:2 and 1:5 with saline, under similar circumstances had no inhibitory influence as measured by spreading activity. If, however,

the incubation period of hyaluronidase with the 1:2 and 1:5 dilutions of serum was increased to 20 and 50 minutes respectively, serum inhibitory activity (approximately 75 per cent) is demonstrable.

Since it had been shown that *in vitro* incubation of undiluted serum with hyaluronidase for 10 minutes produced inactivation of spreading activity, it was of interest to determine whether serum introduced simultaneously with hyaluronidase into the skin without prior *in vitro* incubation, would influence spreading activity. A typical experiment of this type is illustrated in Table III.

TABLE II

*The Effect of in Vitro Incubation of Hyaluronidase with Varying Amounts of Rabbit Serum (Hyaluronidase Concentration Constant)*

Final concentration before injection		Time of <i>in vitro</i> incubation*	Time after injection, ‡ min.				Average I
Hyaluronidase	Serum		2		5		
			A‡	I‡	A	I	
µg./cc.	cc./cc.	min	cm. <sup>2</sup>	per cent	cm. <sup>2</sup>	per cent	per cent
0	0	—	1.64	—	1.84	—	
0.1	0	—	1.79	—	2.01	—	
0.5	0	—	1.89	—	2.35	—	
1.0	0	—	2.67	—	2.94	—	
2.5	0	—	2.99	—	3.70	—	
5.0	0	—	3.43	—	3.76	—	
2.5	0.25	10	2.11	76	2.80	64	70
	0.125	10	3.00	0	3.74	0	0
	0.05	10	2.94	4	3.72	0	2
	0.125	20	2.11	76	2.44	76	76
	0.05	50	1.88	80	2.39	79	80

\* Incubated at 25°C.

† All solutions were injected intradermally in a volume of 0.20 cc.

‡ Area of spread in cm.<sup>2</sup>.

|| Inhibition produced by *in vitro* incubation, in per cent, as evaluated from dosage-response curves.

In this experiment 2, 5, or 10 μg. of hyaluronidase in a volume of 0.2 cc. was added to 0.5 cc. of indicator solution; 0.3 cc. of rabbit serum was then added, the solution mixed, and immediately thereafter 0.2 cc. of the final solution injected. Although the serum was in contact with the hyaluronidase for perhaps as long as 1 minute before the mixture was injected (time required to mix the solutions, load the syringe, etc.) the time of *in vitro* incubation has been taken as zero. For comparative purposes, results obtained by incubating similar concentrations of serum and hyaluronidase for 15 minutes *in vitro*, prior to injection, are likewise shown in Table III. The results of the above experiments were compared with those obtained by injecting hyaluronidase in the absence of serum.

Table III illustrates the inability of serum to affect hyaluronidase spreading activity when both materials are injected almost immediately after mixing, in

contrast to the significant inhibition produced by *in vitro* incubation prior to injection. This result is not due to a possible effect of the indicator affecting the serum inhibition for it was observed that the *in vitro* reaction between serum and hyaluronidase is not significantly influenced by the presence or absence of the hemoglobin indicator during the course of the reaction. The finding that serum does not affect spreading activity when injected together with hyaluronidase, under conditions of minimal prior incubation could be explained in either of two ways: (a) the rate of reaction of the serum factor upon hyaluronidase is slow as compared to the rate of hyaluronidase action upon the hyaluronic acid component of the dermal barrier, or (b) the reaction between serum and hyaluronidase does not take place *in vivo* in skin. The former view is supported

TABLE III

*The Effect of the Simultaneous Injection of Rabbit Serum and Hyaluronidase without in Vitro Incubation*

Final concentration before injection		Time of <i>in vitro</i> incubation*	Time after injection, <i>min.</i>			Average I
Hyaluronidase	Serum		1	2	5	
			I†	I	I	
μg./cc.	cc./cc.	min.	per cent	per cent	per cent	per cent
2.0	0.3	0	2	0	0	1
		15	90	78	85	84
5.0	0.3	0	0	0	0	0
		15	95	88	88	90
10.0	0.3	0	—	0	0	0
		15	—	90	94	92

\* Incubated at 25°C.

† Inhibition produced by addition of serum to hyaluronidase, in per cent, as evaluated by dosage-response curves determined in the same animal.

by recent viscometric work of Hadidian (9) who has found that the addition of hyaluronic acid (prepared from human umbilical cord) to a system containing testis hyaluronidase and serum, completely prevents the serum inhibition of hyaluronidase viscosity-reduction activity usually obtained during the first two minutes of the reaction and significantly decreases the inhibitory activity of the serum upon hyaluronidase ten minutes after initiation of the reaction. These viscometric results obtained by Hadidian, taken in conjunction with the fact that the spreading reaction produced by hyaluronidase is 75 to 80 per cent complete within two minutes (4) could explain the ineffectiveness of the serum antihyaluronidase to influence the spreading reaction of hyaluronidase when both are injected together with a minimal period of prior *in vitro* incubation.



It is also possible that the reaction between serum and hyaluronidase under *in vitro* conditions does not lead to significant inhibition of spreading activity. This latter possibility was tested in the next section of this paper.

*Effect of in Vivo Incubation of Serum with Hyaluronidase.*—The ability of the serum factor to react with hyaluronidase *in vitro* was tested by incubating hyalu-

TABLE IV

*The Ineffectiveness of Serum to Inhibit Hyaluronidase Spreading Activity under Conditions of in Vivo Incubation in Skin*

Initial injection (0.1 cc.) contains		Period of in vitro Incuba- tion*	Interval between 1st and 2nd injections	Time after injection of 1.0 cc. indicator, min.								Average H
Hyal- uronid- ase	Serum			1		2		5		10		
				A: cm. <sup>2</sup>	H: μg.	A cm. <sup>2</sup>	H μg.	A cm. <sup>2</sup>	H μg.	A cm. <sup>2</sup>	H μg.	
μg.	cc.	min.	min.	cm. <sup>2</sup>	μg.	cm. <sup>2</sup>	μg.	cm. <sup>2</sup>	μg.	cm. <sup>2</sup>	μg.	μg.
0	0	—	3	3.18	—	4.12	—	4.65	—	5.25	—	—
0.1	0	—	3	4.13	—	5.34	—	5.97	—	7.52	—	—
0.5	0	—	3	5.00	—	6.10	—	8.60	—	9.65	—	—
1.0	0	—	3	6.85	—	8.90	—	12.32	—	12.70	—	—
2.5	0	—	3	6.53	—	8.70	—	12.00	—	12.83	—	—
1.0	0.09	15	3	3.66	0.1	4.48	0.1	6.32	0.1	7.65	0.1	0.10
1.0	0.09	0	30	6.02	0.7	7.23	0.6	8.00	0.4	10.12	0.6	0.57
	0	0		5.90	0.7	6.77	0.5	7.88	0.4	9.85	0.6	0.55
1.0	0.09	0	65	6.55	0.9	7.35	0.6	8.55	0.5	10.10	0.6	0.65
	0	0		5.60	0.6	7.27	0.6	7.88	0.4	9.90	0.6	0.55
1.0	0.09	0	120	6.85	1.0	7.35	0.6	8.73	0.5	9.80	0.6	0.67
	0	0		6.45	0.9	7.00	0.6	8.57	0.5	9.80	0.6	0.65

\* Incubated at 25°C.

† A, area of spread in cm.<sup>2</sup> of the indicator.

‡ H, is the hyaluronidase equivalent, to the closest 0.1 μg., as obtained from the dosage-response curves.

ronidase with serum in skin for varying intervals of time and then measuring residual hyaluronidase spreading activity. Table IV shows the results of a typical experiment of this series.

Concentrations of hyaluronidase ranging from 1 to 25 μg. per cc. were injected intradermally without indicator in a volume of 0.1 cc. The needle hole and initial bleb were marked with india ink, and 3 minutes later, 1.0 cc. of methemoglobin indicator solution was injected directly

into the previously treated area. The area of spread of the indicator 1, 2, 5, and 10 minutes after injection was measured. On the basis of these results, an amount of hyaluronidase equivalent to 1  $\mu$ g. in 0.1 cc. was selected for *in vivo* incubation with serum. Thus, 0.1 cc. of a 100  $\mu$ g. per cc. solution of hyaluronidase was mixed with 0.9 cc. of rabbit serum, and immediately thereafter, 0.1 cc. volumes of this mixture were injected intradermally. After time intervals ranging from 30 to 120 minutes, these areas were reinjected with 1.0 cc. of indicator solution and the rate of spreading determined in the usual manner. The spreading reactions observed in the areas previously treated with hyaluronidase plus serum, were compared with those obtained by injecting areas which had been treated with 0.1 cc. containing 1  $\mu$ g. of hyaluronidase alone 30 to 120 minutes previously. The *in vitro* antihyaluronidase activity of the serum used in these experiments was checked in the following manner: 0.1 cc. of hyaluronidase (containing 10  $\mu$ g.) was incubated for 15 minutes at 25°C. with 0.9 cc. serum; 0.1 cc. of this mixture was then injected into the skin, and 3 minutes later this area was reinjected with 1.0 cc. of indicator solution.

It will be seen from Table IV that serum incubated *in vivo* with hyaluronidase for periods ranging from 30 to 120 minutes had no inhibitory activity; but that the same concentrations of serum and hyaluronidase incubated *in vitro* for only 15 minutes produced marked inhibition of spreading activity (approximately 90 per cent). It should be emphasized that in three other experiments with rabbit serum the ineffectiveness of the serum factor to inhibit hyaluronidase on *in vivo* incubation was consistently observed, despite the fact that the period of *in vivo* incubation in skin in some experiments was as long as 5 hours. These results indicate that in the *in vivo* environment of skin, the reaction between serum antihyaluronidase and hyaluronidase, either does not occur, or if a reaction does take place, it is readily reversible.

#### DISCUSSION

The results obtained in this study demonstrate that the *in vitro* reaction between normal rabbit or pig serum and hyaluronidase is profoundly modified by the *in vivo* conditions that exist in rabbit skin. Serum incubated *in vivo* with hyaluronidase in skin for as long as 5 hours, fails to produce a significant inhibitory effect, although marked inhibition of hyaluronidase activity is demonstrable when similar concentrations of serum and hyaluronidase are incubated *in vitro* for 10 to 15 minutes. This disparity between the *in vivo* and *in vitro* effectiveness of serum to inhibit hyaluronidase spreading activity, indicates that environmental conditions in the skin are unfavorable for the reaction between the serum inhibitor and hyaluronidase which regularly occurs *in vitro*.

While it is possible to explain these results on the basis that the serum factor is inactivated in skin or eliminated, an alternative explanation seems equally likely. Recent studies by Hadidian and Pirie (10) using the viscometric method, clearly demonstrate that the inhibition of hyaluronidase by serum is not an enzymic reaction, but is due to a reversible binding of the enzyme activity by the serum factor. Alterations of various conditions (salt and phos-

phate concentration, etc.) modify the rate of the inhibitory binding reaction as well as the rate of the release of hyaluronidase from its inactive form. It therefore seems possible that factors in skin could either inhibit the serum binding of hyaluronidase, or increase the dissociation of the hyaluronidase bound by serum. One factor operative *in vivo*, which could function in the former manner, is dermal hyaluronic acid; for it has been shown using the viscometric technic that hyaluronic acid significantly decreases the rate of reaction of the serum factor upon hyaluronidase, presumably by competitive inhibition (9).

Although it is not possible at this time to completely explain the ineffectiveness of the serum factor under *in vivo* conditions in skin, the results of this study would nevertheless appear to have significance concerning the rôle of the serum inhibitor factor in those invasive processes catalyzed by hyaluronidase. Thus the results of this study cast considerable doubt upon the biological significance of the enzymatic theory for the mechanism of invasion recently proposed by Haas (3). By extrapolation of viscometric data to bacterial invasiveness *in vivo*, Haas has developed the concept that the serum inhibitory factor ("antinvasin I") represents the body defense mechanism against bacterial, and other, invasions facilitated by hyaluronidase. According to his view, "proinvasin I," a factor found in bacteria and snake venoms, destroys "antinvasin I" and thus facilitates the invasive process. Other enzymes, "antinvasin II," and possibly "proinvasin II" and "antinvasin III" participate in the manner indicated by their names (3). In substance this theory views the process of invasion in skin, as the resultant of enzymatic activities present in the invading agent and in the body defense mechanism. Without considering the question as to whether the activities described by Haas, are, in fact, enzymatic, our results which demonstrate that hyaluronidase inactivation by "antinvasin I" does not occur *in vivo* would seem to remove "antinvasin I" from the position of representing the body defense mechanism against invasions facilitated by hyaluronidase. It is of interest to mention that having removed the "antinvasin I" factor from the Haas theory of invasion, the physiological significance of the subsequent factors in the scheme ("proinvasins I and II" and "antinvasin II and III") disappears because all of these are believed indirectly to affect hyaluronidase *via* "antinvasin I."

The results of these studies throw no light on the *in vivo* function of the anti-hyaluronidase factor of serum and further work will be necessary to elucidate the biological rôle of this factor.

#### SUMMARY

The reaction between normal serum and hyaluronidase has been studied *in vitro* and under *in vivo* conditions in skin. Using *in vitro* conditions of incubation, serum exhibits antihyaluronidase activity as measured by assay of hyaluronidase spreading activity in skin. This confirms the work of others, who

have previously described the serum inhibitory factor using other tests of hyaluronidase activity. When, however, hyaluronidase and serum are allowed to incubate in skin under *in vivo* conditions, no inhibitory influence of serum upon hyaluronidase spreading activity is evident. This latter finding has been taken to indicate that the environmental conditions in skin are unfavorable for the inhibitory reaction of serum upon hyaluronidase. The disparity between the *in vivo* and *in vitro* effectiveness of serum, and the significance of the serum factor as a defense mechanism against invasive processes, have been briefly discussed.

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# TOLERANCE TO BACTERIAL PYROGENS

## I. FACTORS INFLUENCING ITS DEVELOPMENT\*

By PAUL B. BEESON, M.D.

WITH THE TECHNICAL ASSISTANCE OF ELIZABETH ROBERTS

(From the Department of Medicine, Emory University School of Medicine, and the Medical Service, Grady Hospital, Atlanta)

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Many different bacteria produce substances which, when administered parenterally to human beings or to experimental animals, cause elevation of the body temperature. These pyrogens are of some importance in clinical medicine, because their presence in materials injected for therapeutic purposes may cause severe pyrexial reactions in patients. On the other hand bacterial pyrogens are sometimes administered intentionally, to induce fever, as in the treatment of neurosyphilis by intravenous injections of typhoid vaccine.

Pyrogenic activity is a property shared by many different bacteria, although generally it is quantitatively greater in Gram-negative and bacillary forms than in Gram-positive or coccal forms. The pyrogenic fractions themselves are relatively stable to heat; ordinary sterilization in the autoclave does not inactivate them. Chemical investigation of these fractions has produced evidence indicating that they are complex carbohydrates (1-3). Some of these purified fractions have been antigenic while others have not. All such fractions are potent toxins, less than a milligram may cause death of a rabbit. Such knowledge as we have of their mode of injury indicates that there is widespread damage to capillaries and larger blood vessels (4). Additional evidence of this type of injury is found in the fact that the pyrogenic fractions of certain bacteria can elicit the Schwartzman phenomenon (5), and can cause hemorrhagic necrosis of mouse sarcoma (6).

The amount of purified bacterial pyrogen necessary to produce fever may be very small. As little as 0.5 microgram of a fraction from *Serratia marcescens* is sufficient to cause high fever in a rabbit (7). The mechanism by which this fever is produced has not been elucidated, but one significant feature is the latent period which is always observed between the time of the intravenous injection of the pyrogen and the beginning of the rise in body temperature. In human beings the time lag is usually between 45 and 90 minutes, while in rabbits it is usually 15 to 30 minutes. The existence of a latent period seems to indicate that the pyrogen does not act by a direct effect upon the temperature-regulating centers in the brain.

It is well known to clinicians that when patients are being given intravenous injections of typhoid vaccine as a method of fever therapy, the dose of vaccine has to be increased at successive treatments in order to bring about comparable temperature elevations. Some patients, after 8 or 10 bouts of fever, may re-

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quire as much as 250 ml. of typhoid vaccine for a single day's treatment (8). No satisfactory explanation of the mechanism of this remarkable tolerance has been given. The present communication describes a series of experiments done on rabbits to determine some of the factors which influence its development. A preliminary report has previously been published (9).

### Materials and Methods

The pyrogenic materials employed were: (1) *Eberthella typhosa* vaccine.<sup>1</sup> This vaccine is ordinarily used for human immunization and fever therapy. The density of the bacterial suspension is approximately one billion organisms per ml. Microscopic examination shows few intact bacillary forms, the gross turbidity being due largely to cellular debris. (2) *S. marcescens* vaccine. The organism was obtained from the American Type Culture Collection. It was cultivated in tryptose phosphate broth at 37°C. for 4 days, then separated by centrifugation, suspended in physiologic salt solution, and killed by heating at 100°C. for 5 minutes. The density of the final suspension was approximately equal to that of the typhoid vaccine. (3) *Pseudomonas aeruginosa* filtrate. The organism was isolated in this laboratory, from the urine of a patient with pyelonephritis. A culture filtrate was prepared, following the procedure used by Welch *et al.* (10) in preparing a standard pyrogen for the First U. S. P. Collaborative Study of Pyrogens. (4) Purified *E. typhosa* pyrogen (2).<sup>2</sup> (5) Purified *S. marcescens* pyrogen (1).<sup>3</sup>

With each pyrogen a dose was determined which would elicit a marked febrile reaction but which would not cause death of the animal. In the case of typhoid vaccine, which was used in most of the experiments, the amount found to be suitable was 1 ml. of a 1:8 dilution. This is approximately 100 times the quantity necessary to produce a definite rise in body temperature in rabbits of this size. It has been given to more than 300 rabbits without a fatality. With the other 4 pyrogens, doses which elicited similar febrile responses were: *S. marcescens* vaccine, 1 ml. of 1:8 dilution in physiologic salt solution; *Ps. aeruginosa* filtrate, 0.5 ml.; purified *E. typhosa* pyrogen, 30  $\mu$ g. in 1 ml. physiologic salt solution; purified *S. marcescens* pyrogen, 10  $\mu$ g. in 1 ml. physiologic salt solution.

The physiologic salt solution used was tested at intervals, and was always pyrogen-free. Glassware, syringes, and needles were sterilized by dry heat at 170°C. for 2 hours. This is sufficient to inactivate any pyrogen present.

The rabbits used were males, weighing 2 to 3 kg. Several different breeds were employed, including New Zealand white, hare brown, and Chinchilla. The animals were housed in air-conditioned rooms in metal cages, at a temperature of 70-80°F. During experiments each rabbit was placed in a wooden stall, and secured by a head board. An opening in the floor of the stall permitted insertion and retention of a mercury thermometer without much disturbance of the rabbit. The rectal temperature was taken every 30 minutes. Animals whose temperatures were found to be higher than 103.6° were excluded from the test. Three readings were obtained before injecting a pyrogen, in order to establish a base line. No food or water was given during a test period. In order to avoid excessive fatigue, the animals were never kept in the stalls longer than 8 hours; this limited the observation period following pyrogen injection to 7 hours.

Temperature records of the rabbits were plotted on  $\frac{1}{4}$  inch graph paper, using one vertical line for each degree Fahrenheit and one horizontal line for each half-hour of test.

<sup>1</sup> Obtained from the Laboratories of the Georgia Department of Public Health.

<sup>2</sup> Obtained from Professor J. M. Nelson, Department of Chemistry, Columbia University.

<sup>3</sup> Obtained from Dr. M. J. Shear, National Cancer Institute, Bethesda, Maryland.

In order to obtain a numerical expression of both the height and duration of a fever, a "fever index" was calculated. This was done by taking as a base level the animal's temperature at the time of injecting the pyrogen, and measuring with a planimeter the area enclosed between this line and the course of the elevated temperature. When the temperature failed to return to the base level within the time limit, as occurred frequently with the first injection of pyrogen, in order to define an area for measuring the fever index a vertical line was drawn between the last temperature and the base line. The fever index is expressed directly in terms of vernier units of the planimeter. The instrument used was a Keuffel and Esser compensating planimeter No. 4236.

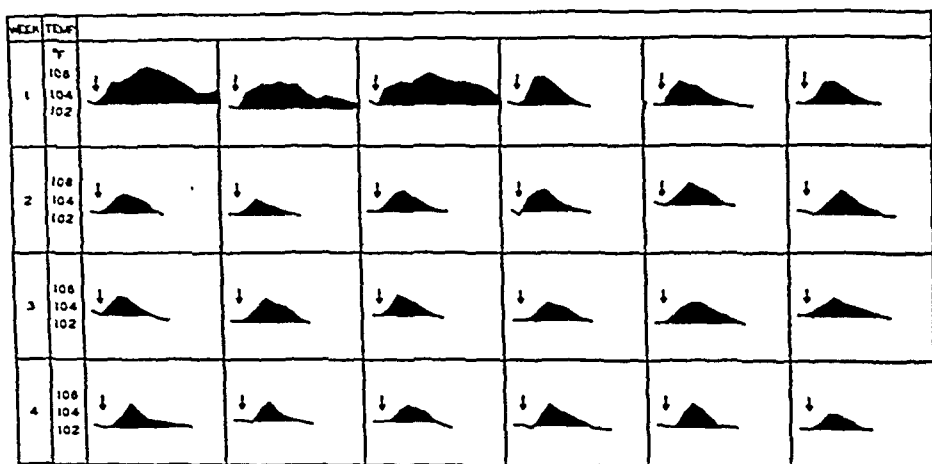


FIG. 1. Febrile reactions of a rabbit in response to daily intravenous injections of  $\frac{1}{8}$  ml. *E. typhosa* vaccine. The chart shows the characteristic marked diminution in febrile reaction at the end of the 1st week, with little change thereafter.

#### EXPERIMENTAL

*Temperature Responses to Repeated Injections of the Same Dose of Pyrogen.*—When animals were given daily injections of the same dose of bacterial pyrogen there was a characteristic pattern of response. The first injection usually caused a rise in body temperature 3–5°F., and the temperature seldom returned to the starting level within the experimental time limit of 7 hours. On the 2nd and 3rd days the febrile responses were nearly as severe as on the first injection, but from the 4th day onward a progressive reduction in pyrexial reaction was nearly always evident. Sometime during the 2nd week a state was reached at which a "minimal" febrile response was elicited, and after that the animal continued to react to each injection with about the same amount of fever; i.e., a rise of 2–3°F., with return to the normal level in from 3 to 5 hours. Fig. 1 illustrates the successive fevers of one rabbit given a series of daily injections of  $\frac{1}{8}$  ml. of typhoid vaccine for a period of 4 weeks.

A collection of observations bearing on this subject is presented in Fig. 2. Here the fever indices of 85 animals are plotted according to the number of

daily injections of  $\frac{1}{8}$  ml. typhoid vaccine received. These comprise all of the observations obtained in the course of several different experiments done during this study, wherein no other modifying factor had been introduced. Temperature elevations were not measured every day in the various experiments; nevertheless it is apparent that the trend of response in this group corresponds to the preceding description.

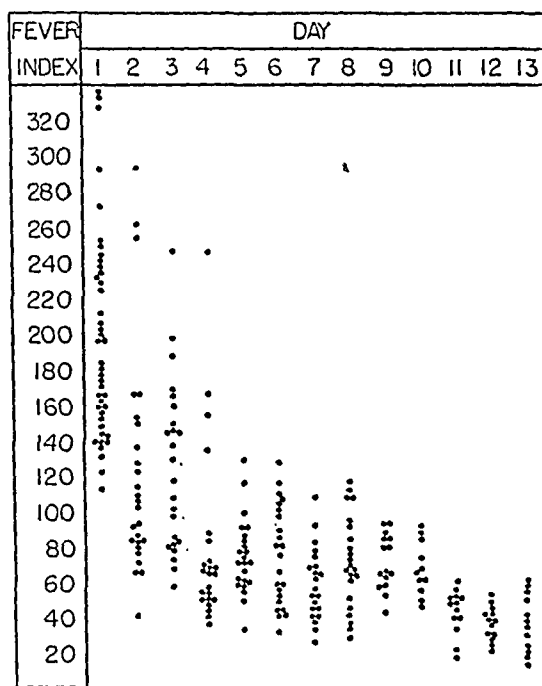


FIG. 2. Fever indices of 85 rabbits, plotted according to number of daily injections of  $\frac{1}{8}$  ml. *E. typhosa* vaccine. These data were obtained in the course of several different experiments. They show the trend to diminished febrile reaction which is observed when injections of pyrogen are given every day.

Repeated injections of *S. marcescens* vaccine, *Ps. aeruginosa* filtrate, or of the purified pyrogens of *E. typhosa* or *S. marcescens* brought out the same type of response, *i.e.* a progressive decline in febrile reaction during the 1st week or 10 days, thereafter a fairly constant "minimal" reaction to each injection.

*Effect of Varying the Interval between Injections.*—When injections of pyrogen were spaced at different intervals of time, it became apparent that the least febrile reaction was obtained when the pyrogenic material was injected every day. Animals injected only once or twice a week exhibited some diminution in their febrile responses, but this was not as marked as could be attained by daily injections of pyrogen. An example is shown in Fig. 3. There it will be observed that in a group of 4 rabbits injected with typhoid vaccine once a week for a period of 14 weeks, there was some diminution in the average fever



index, but that these animals, even after 14 weeks, had not developed a "minimal" response. They showed further reduction when the interval between doses was shortened to 1 day. Fig. 3 also shows the results in another group of 4 animals to which injections of typhoid vaccine were given twice a week for a period of 10 weeks. A series of 20 inoculations brought about a diminution in the febrile reactions, but again there was a further significant reduction when the interval between injections was shortened to 1 day.

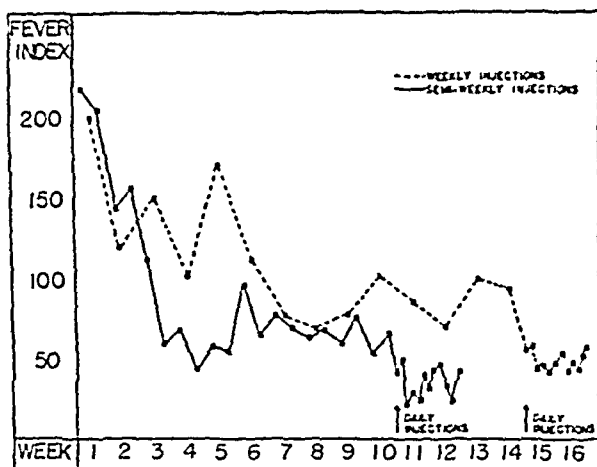


FIG. 3. Average fever indices in 2 groups of 4 rabbits. One of these received injections of  $\frac{1}{2}$  ml. *E. typhosa* vaccine once a week for 14 weeks, then a series of daily injections. The other group received injections twice a week for 10 weeks, then a series of daily injections. The results indicate that the tolerance to pyrogen becomes most highly developed when injections are given every day.

*Loss of Tolerance after Rest Period.*—When animals which had been rendered relatively unresponsive to a given dose of pyrogen were allowed to rest for some days and then retested, it was found that some increase in responsiveness had occurred.

For example, one group of 10 rabbits received  $\frac{1}{2}$  ml. of typhoid vaccine every day for 13 days. The average fever index for the 10 animals on the occasion of their first injection was 196. On the 13th day the average fever index for the same group was 39. Five of them were then rested for 8 days, and the remaining 5 for 22 days. On resuming the injections the fever index was found to have risen in the group rested 1 week to 85; whereas in those rested for 3 weeks the average fever index was 186. It appeared then, that after a rest period of 1 week, some, but not all, of the responsiveness had returned and that after a rest as long as 3 weeks the response was essentially the same as that of normal animals.

*Lack of Correlation with the Titer of Specific Typhoid Agglutinins.*—The level of serum agglutinins for the typhoid vaccine did not appear to be closely related to the febrile responses. In the preceding experiment the average titer

of antibodies in the rabbits which were rested for 1 week was 1:2560, while that of the rabbits rested for 3 weeks was 1:320. While there had been an appreciable reduction in titer at 3 weeks, the 4 animals still had considerable amounts of agglutinin in their blood, yet on reinjection of typhoid vaccine they gave a febrile response essentially like that of normal animals.

*Effect of Reducing the Dose of Pyrogen.*—Another experiment which demonstrated the rapid loss of responsiveness to pyrogens is illustrated in Fig. 4.

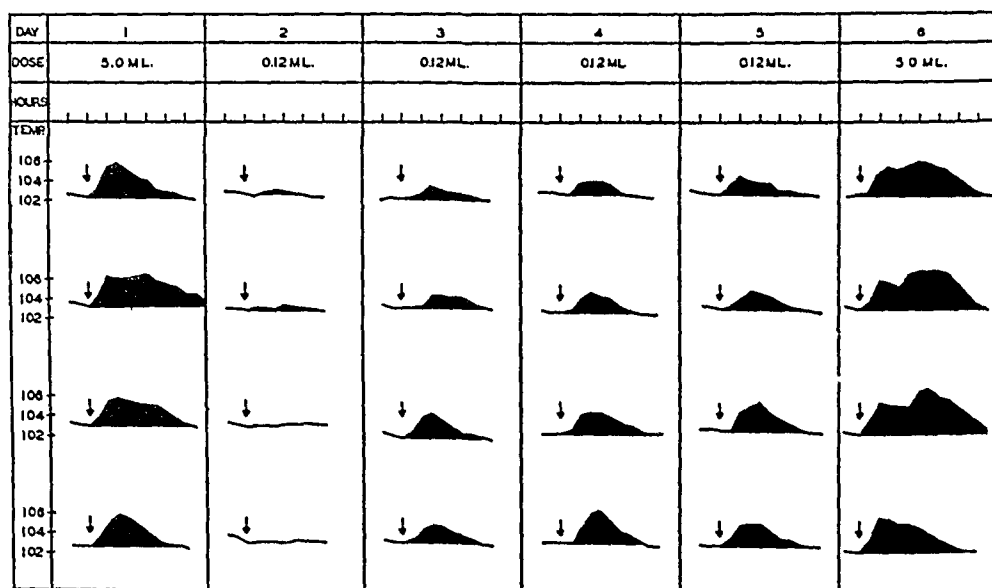


FIG. 4. Successive daily fevers in 4 rabbits during a period when the dosage of pyrogen was abruptly changed twice. During 2 weeks prior to the period charted each rabbit had been given increasing doses of *E. typhosa* vaccine, up to 5.0 ml. The left-hand column shows their febrile reactions to this large dose. On the 2nd day the dose was reduced to  $\frac{1}{8}$  ml. Note that there was little or no febrile reaction. Three more daily injections of  $\frac{1}{8}$  ml. caused higher fevers. On the 6th day a dose of 5.0 ml. was given again with the production of more fever than on the first charted day.

Four rabbits had received increasing doses of typhoid vaccine, beginning with  $\frac{1}{8}$  ml., and progressing to 5 ml. at the end of 2 weeks. Their temperature responses to this large dose are reproduced in the first column of this figure. On the following day, when the dose was reduced to only  $\frac{1}{8}$  ml., the animals reacted with little or no rise in temperature. The next day, injection of  $\frac{1}{8}$  ml. produced slight but definite fever in all of them, and thereafter the response to  $\frac{1}{8}$  ml. was similar to that of animals which had received a long series of daily inoculations with that quantity of pyrogen, as can be seen by comparison with Fig. 1. Finally, 5.0 ml. were given again. This induced somewhat more fever than on the first occasion, indicating that the reduced daily dose did not maintain a maximum tolerance.

This experiment was repeated on two occasions, with similar findings.

These results indicate that the animals are least responsive on the day after an injection, and that they become more responsive as early as the 2nd or 3rd day.

*Specificity of the Tolerance for Different Bacterial Pyrogens.*—When animals which had been rendered unresponsive to one bacterial pyrogen were tested with a different one, they were found to be somewhat tolerant of it as well.

For example, 5 rabbits were given 10 daily injections of  $\frac{1}{2}$  ml. of *S. marcescens* vaccine and then received  $\frac{1}{2}$  ml. of typhoid vaccine. The fever indices obtained were 98, 69, 98, 65, and 85. Comparison of these with first day responses of normal animals to that dose of typhoid vaccine, shown in Fig. 2, indicates that all were below the ordinary range. In another experiment, 8 rabbits received 10 daily injections of  $\frac{1}{2}$  ml. of *Ps. aeruginosa* filtrate, and then were given  $\frac{1}{2}$  ml. of typhoid vaccine. All showed a considerable reduction in febrile response as compared with normal animals; the fever indices being 76, 70, 74, 74, 71, 62, 66, and 70.

*Passive Transfer of Tolerance to Pyrogens.*—An attempt was made to confer the unresponsive state by injection of serum from highly tolerant animals.

Three rabbits were given daily injections of typhoid vaccine in increasing amounts up to 5.0 ml. On the day following the last injection, when a high degree of tolerance would be expected, the 3 animals were bled by cardiac puncture, and their sera were pooled. This pooled serum was then given to 6 normal rabbits, in doses of 10 ml., intravenously. One hour later the recipient animals were given  $\frac{1}{2}$  ml. of typhoid vaccine. Six control rabbits were treated with normal rabbit serum and then with typhoid vaccine, in the same quantities. The febrile responses of all 12 rabbits fell within the range of ordinary responses (see first day responses, Fig. 2); and there was no appreciable difference in the fevers in the two groups.

*Effect of Preventing Temperature Elevations during Development of the Tolerance.*—

Eight rabbits were given daily injections of  $\frac{1}{2}$  ml. of typhoid vaccine. The febrile reactions to these injections were prevented by administering 0.6 gm. amidopyrine through a stomach tube, 1 hour previous to each injection of pyrogen. On the 8th day the premedication with amidopyrine was omitted. At this time, injection of the same dose of typhoid vaccine caused temperature elevations characteristic of animals tolerant to the pyrogen, the fever indices being 65, 54, 65, 106, 57, 103, 48, and 56. Reference to Fig. 2 will show that these were below the range of first day responses to this dose of typhoid vaccine.

It seems, then, that elevation of body temperature during the period of immunization is not essential to the development of tolerance to bacterial pyrogens.

*Effect of Mechanically Induced Fever on Tolerance to Pyrogens.*—An experiment was designed to determine the effect of a series of mechanically induced fevers on the temperature response to bacterial pyrogens.

For this purpose a fever cabinet similar to that used by Ellingson and Clark (11) was constructed. Four rabbits were given daily fevers for 8 days, their temperatures being raised rapidly to a level of 106–107.5°F., and maintained in that range for 2 hours each day. One rabbit died during this procedure, apparently of the effects of the pyrexia. On the day after the last of the mechanically induced fevers the 3 remaining animals were given injections of  $\frac{1}{2}$  ml. of typhoid vaccine. They reacted with high prolonged elevations, the fever indices being 191, 206, and 266.

The findings indicate that mechanically induced fever does not induce tolerance to bacterial pyrogens.

*Behavior of Circulating Leucocytes during a Course of Injections.*—In view of the marked fluctuations in circulating leucocytes which are known to result from intravenous injection of bacterial suspensions, leucocyte counts were made on a series of 6 rabbits throughout a 10 day course of injections of  $\frac{1}{8}$  ml. of typhoid vaccine. As would be expected a leucopenia was found during the first hour after pyrogen injection, and this was followed by a leucocytosis in 4 to 8 hours. No striking alteration in this pattern of response was observed during the period of the injections except that the deviations in cell count tended to be smaller toward the end of the experimental period.

*Effect of Repeated Injections of Pyrogens on the General Health.*—Animals which received daily injections of pyrogens, in the quantities mentioned for periods of several weeks showed no sign of deterioration in general health. They tended to gain weight, their coats remained sleek, and there was no special tendency to develop intercurrent infections.

#### DISCUSSION

The experiments show clearly that rabbits given repeated injections of bacterial pyrogens become relatively unresponsive to the fever-promoting action of these materials. This is in accord with clinical observations on human beings undergoing fever therapy with typhoid vaccine. Such an alteration in temperature response of experimental animals has apparently not been observed by certain laboratory workers, since several recent reports of work with bacterial pyrogens indicate that animals have been used in tests repeatedly, sometimes daily, and that the possibility of alteration in reactivity has not been considered (12-14). Seibert, who was one of the first to demonstrate that pyrexial reactions to therapeutic intravenous injections were usually due to pyrogen contamination of the fluid (15), stated that in her experience rabbits never showed any indication of an immunizing or a sensitizing effect. This conclusion was supported later by Banks (16). A collaborative study of this matter was inaugurated by the Committee of Revision of the U. S. Pharmacopeia, in 1941 (17). A series of 3300 tests was made on 253 rabbits, a potent pyrogen (*Ps. aeruginosa* filtrate) being given to each animal twice weekly for 5 weeks. It was found that there was some lessening in the average height of the febrile reaction toward the end of the 5 week period. Our findings make it seem probable that those workers would have obtained more clear cut differences had the pyrogen injections been given daily. Another factor which explains in part the difference between our findings and those of other workers is that our calculations have included not only the height but also the duration of each fever. A characteristic of the immune response is the more rapid return of the temperature to the starting level (see Fig. 1).

The following evidence may be cited to indicate that the production of specific humoral antibodies may have little or no relation to the development of tolerance to pyrogens.

1. Animals lost their tolerance to typhoid pyrogen after a rest period of 3 weeks, that is to say at a time when specific agglutinins were still found in their sera.

2. The tolerance evidenced to one bacterial pyrogen obtained in some degree to pyrogens of bacteria not serologically related.

3. Passive transfer of the tolerance could not be accomplished.

4. The purified typhoid pyrogen, a carbohydrate material, which, by our tests, did not have antigenic properties, proved capable of inducing the same type of insusceptibility as vaccines of whole bacteria. Some observations of other workers support this evidence. Welch and associates showed that the pyrogenic fraction from *Ps. aeruginosa* was not antigenic (10). Favorite and Morgan studied the effects of a pyrogenic fraction from *E. typhosa* in human beings, and noted that "the titer of circulating antibody did not seem to be closely related to the development of tolerance to the toxicity of the antigen" (18).

#### SUMMARY

In a study of the febrile responses of rabbits to repeated intravenous injections of pyrogenic substances from *Eberthella typhosa*, *Serratia marcescens*, and *Pseudomonas aeruginosa*, the following observations were made:

1. A characteristic pattern of response to daily injections of the same dose of pyrogenic material was noted. This consisted of a progressive diminution in febrile response during the 1st week or 10 days, after which an animal responded to each injection with approximately the same degree of fever, even when the injections were continued for several weeks.

2. Animals given injections of the same amount of pyrogenic material at semiweekly or weekly intervals showed some diminution in febrile reaction but the alteration was less pronounced than that in animals injected every day.

3. Pyrogen tolerance appeared to be lost quickly. Animals allowed to rest for approximately 3 weeks reacted to readministration of pyrogen with fever comparable with that which occurred after the first injection.

4. By gradually increasing the size of the daily dose of pyrogen a tolerance could be established such that a reduced, but still considerable, amount of pyrogen caused no fever whatever.

5. Rabbits that had been injected with *S. marcescens* or *Ps. aeruginosa* pyrogens showed a diminished febrile response to *E. typhosa* vaccine.

6. Passive transfer of the unresponsiveness to pyrogens could not be demonstrated.

7. Prevention of temperature elevations during the course of immunization

by use of an antipyretic drug did not interfere with the development of tolerance to pyrogens.

8. A series of mechanically induced bouts of fever did not reduce the responsiveness to bacterial pyrogens.

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# TOLERANCE TO BACTERIAL PYROGENS

## II. RÔLE OF THE RETICULO-ENDOTHELIAL SYSTEM\*

By PAUL B. BEESON, M.D.

With the Technical Assistance of ELIZABETH ROBERTS

(From the Medical Service, Grady Hospital, and the Department of Medicine, Emory University School of Medicine, Atlanta)

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The preceding article (1) describes a series of observations on the tolerance which develops as a result of repeated intravenous injections of bacterial pyrogens, and which is characterized by a diminished febrile reaction to these substances. This state appears to be largely independent of serologic specificity; it is also remarkable for its short duration. The present article deals with experiments designed to elucidate the mechanism by which it develops, with special reference to the rôle of the reticulo-endothelial (R-E) system.

### *Materials and Methods*

Tests were conducted under the conditions described in the preceding article. Two agents were employed for R-E blockade: 25 per cent colloidal thorium dioxide (thorotrast, Heyden Co.), and 1 per cent aqueous trypan blue. The dose of thorotrast was 9 ml.; this was given intravenously 16 hours before the test injection of pyrogen. The dose of trypan blue was 6 ml.; this was given twice, 16 hours and 1 hour previous to injection of the pyrogen. In experiments on the effect of R-E blockade the doses of pyrogens used were the same as those given in the preceding article. In tests of the speed of removal of pyrogen from the blood, the donor animal was prepared for cardiac puncture. The pyrogenic material was then injected into an ear vein, at a rate of 1 ml. in 5 seconds. Exactly 4 minutes after the conclusion of the injection, the heart was punctured, and 8 ml. of blood drawn rapidly into a syringe containing 2 ml. of 3.8 per cent sodium citrate. The citrated plasma was separated immediately by centrifugation, and was injected into a test animal within 1 hour of the time of the bleeding. The technic for asepsis was observed at all stages of the procedure. The amounts of pyrogens given to the donor animals were: *Eberthella typhosa* vaccine, 5 ml.; *Serratia marcescens* vaccine, 3 ml.; *Pseudomonas aeruginosa* filtrate, 5 ml.; purified *Eberthella typhosa* pyrogen, 0.5 mg. in 5 ml. saline; purified *Serratia marcescens* pyrogen, 1.0 mg. in 5 ml. saline.

### EXPERIMENTAL

*Effect of R-E Blockade on the Tolerance.*—Rabbits were given daily injections of one of the pyrogen preparations until a marked diminution in febrile response had developed; i.e., for 6 to 10 days. They then received intravenous injections of a blocking agent, after which the same dose of pyrogen was administered. Under these circumstances there was a marked increase in the febrile reaction. In most instances the febrile reaction following blockade was even greater than

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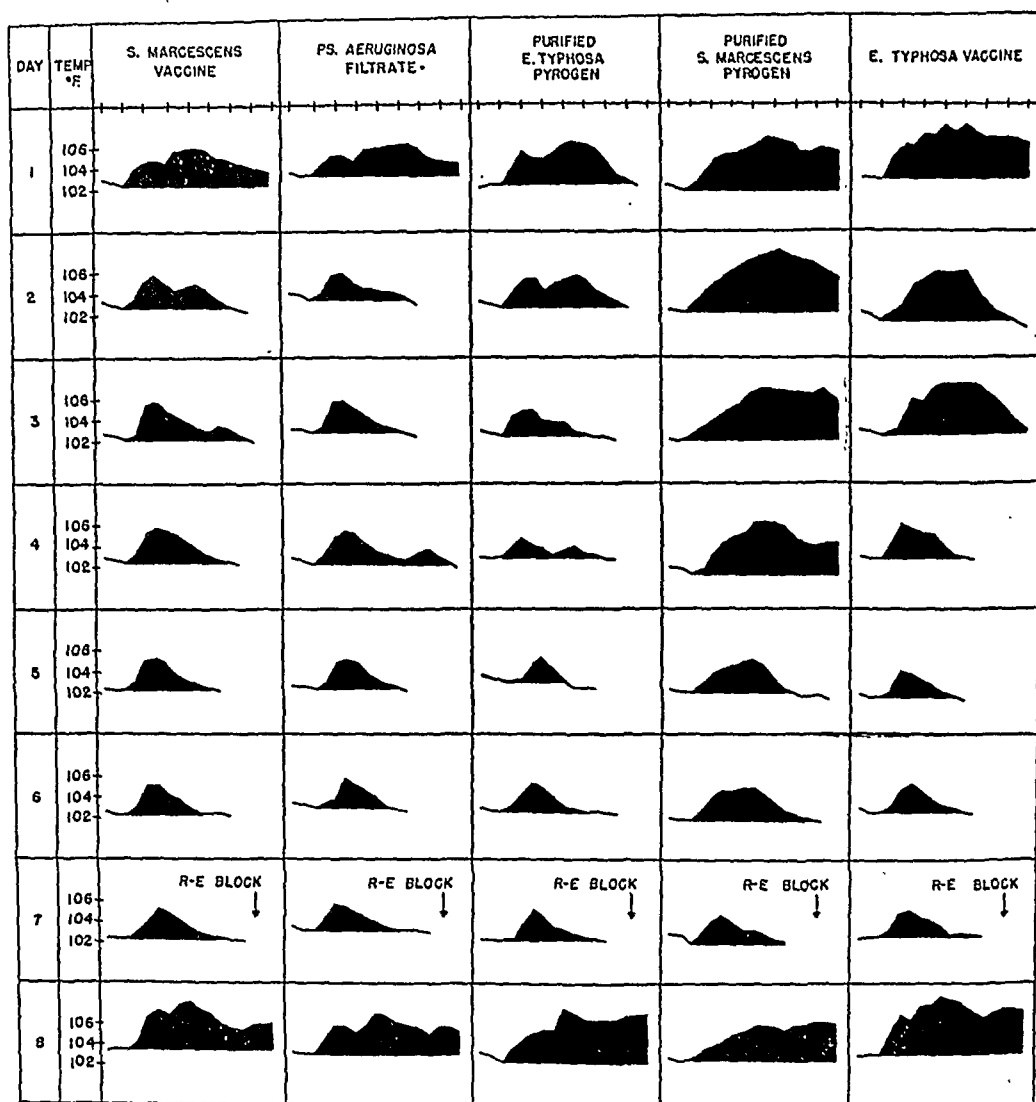


FIG. 1. Effect of R-E blockade on febrile responses of pyrogen-tolerant rabbits. Each column shows the daily febrile response of a rabbit to a constant dose of one of the pyrogens. By the end of 7 days all had a considerable reduction in febrile response. At this time thorium dioxide was given to produce R-E blockade. On the following day the febrile reaction to the pyrogen was much greater.

that which occurred after the first injection of the pyrogen. Experiments of this type were carried out on 64 rabbits, and without exception the fever was greater after R-E blockade than before. Fig. 1 shows a typical example, with each of the 5 different pyrogen preparations, using thorotrast as the blocking agent. The effect of thorotrast was usually quantitatively greater than that of trypan blue, although the latter agent invariably was responsible for a marked increase in the febrile reaction.



*Speed of Disappearance of Pyrogen from the Blood.*—In view of the finding that R-E blockade appeared to abolish the induced tolerance for pyrogens, the possibility was considered that tolerance might be determined by the efficiency of the mechanism for removing pyrogen from the circulating blood. Accordingly, experiments were carried out in which blood samples were taken from normal animals and those with induced tolerance a few minutes after they had received large doses of pyrogen. These samples were then tested for pyrogen content, by measuring their effect in causing temperature elevations in other

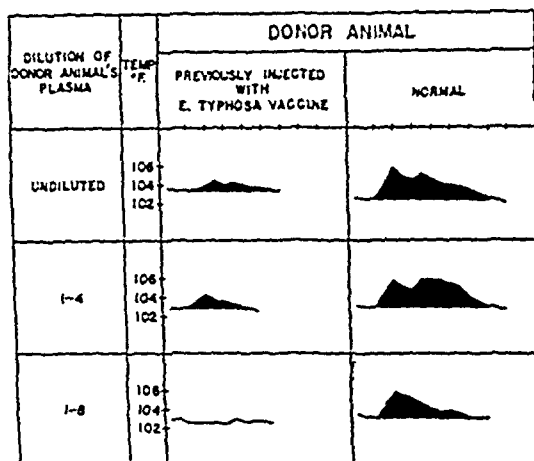


FIG. 2. Effect of previous injections of *E. typhosa* vaccine on speed of disappearance of the pyrogen from the blood. This shows the relative pyrogenic activity of plasma from an animal so treated, and a normal rabbit 4 minutes after each had received 5.0 ml. *E. typhosa* vaccine. Different dilutions of each sample were given to 3 normal rabbits.

rabbits. Preliminary tests on normal animals showed that, even after administration of large doses of pyrogen, little trace could be detected in the circulating blood 8 to 10 minutes later. At 4 to 6 minutes, however, pyrogenic activity was always considerable.

An animal was given increasing quantities of *E. typhosa* vaccine during a period of 2 weeks, up to a dose of 5 ml. daily. On the 15th day this quantity was given and the animal was tested for capacity to remove it from the blood, in comparison with a normal animal of the same breed and size. The experimental technique has already been described. Fig. 2 shows the pyrogenic potency of the blood of a rabbit treated as just described and of a normal rabbit 4 minutes after each had received 5 ml. of *E. typhosa* vaccine intravenously. Three normal animals were used in testing the blood of each rabbit receiving the pyrogen. It will be observed that a considerable difference in pyrogenic activity was found. With the undiluted samples and with the 1-4 dilutions there was a marked difference in febrile responses of the test rabbits, and with

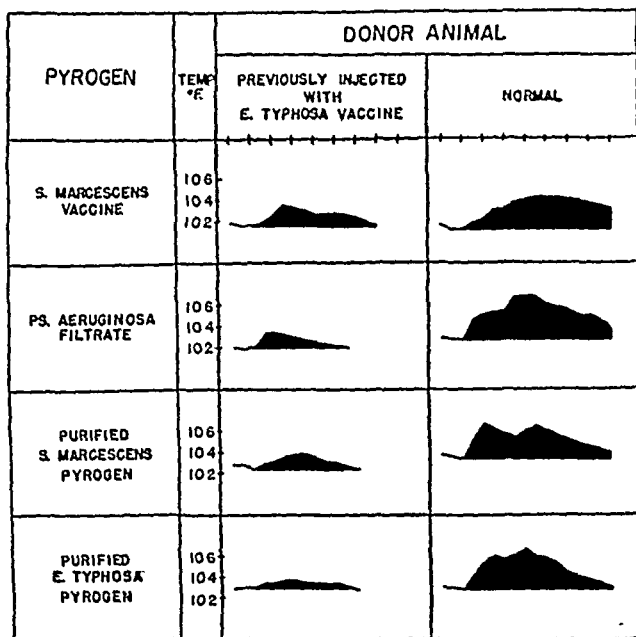


FIG. 3. Effect of previous injection of *E. typhosa* vaccine on ability to remove different pyrogens from the blood. This shows comparative febrile reactions of normal rabbits to plasma from tolerant and normal donor rabbits. Donors had been given large doses of pyrogen 4 minutes before plasma samples were obtained.

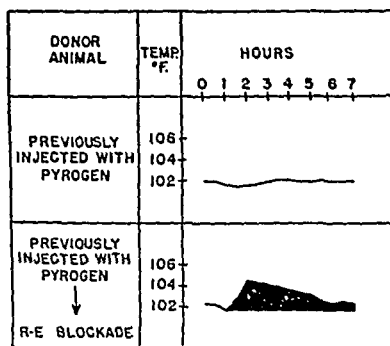


FIG. 4. Effect of R-E blockade on ability of previously tolerant animal to remove pyrogen from the blood. This shows the pyrexial reactions of normal rabbits to plasma samples from 2 rabbits taken 4 minutes after each had received 5.0 ml. *E. typhosa* vaccine. One of these donor animals had received thorium dioxide on the previous day.

1-8 dilutions the sample from the previously injected donor animal caused practically no temperature elevation, whereas that from the normal animal still contained sufficient pyrogen to cause a considerable pyrexial reaction.

Similar experiments were carried out, testing the speed of disappearance of other pyrogens from the blood. It was found that repeated injections of *E.*

*typhosa* vaccine created a state such that the pyrogens of *S. marcescens* and *Ps. aeruginosa* disappeared from the circulating blood more quickly than was the case with normal animals. Fig. 3 presents examples of such experiments, using the 4 other pyrogen preparations employed in this work: *S. marcescens* vaccine, *Ps. aeruginosa* filtrate, purified *S. marcescens* pyrogen, and purified *E. typhosa* pyrogen. As is shown, the amount of pyrogenic material remaining in the circulation 4 minutes after injection was always less in treated than in normal animals.

*R-E Blockade and the Speed of Disappearance of Pyrogen from the Blood.*—A study was made of the effect of R-E blockade on the rate of disappearance of pyrogen from the circulating blood. Two rabbits were given daily injections of *E. typhosa* vaccine in increasing doses up to 5 ml., during a period of 2 weeks. One of them was then given thorotrast as a blocking agent, and on the following day both animals were tested for speed of removal of the same dose of *E. typhosa* vaccine pyrogen from the blood. It was found that the animal given R-E blockade had more circulating pyrogen 4 minutes after the injection than did the other tolerant animal. Four experiments of this kind were done, with the same result; one of them is illustrated in Fig. 4.

#### DISCUSSION

Explanation of the mechanism of tolerance for pyrogens is hampered by the inadequacy of our knowledge regarding the pathogenesis of fever. Clinical experience has demonstrated that fever is a sensitive index of many kinds of pathologic process, whose only common factor is injury of tissue. It is probable that the temperature elevation produced by bacterial pyrogens is also a reflection of injury, and not due to a direct effect on temperature-regulating centers in the brain. This view is supported by the fact that there is always a time lag between the injection of the pyrogen and the beginning of the rise in temperature, and by the fact that purified pyrogens are lethal toxins (2).

The experimental evidence which has been presented here indicates that the functional state of the R-E system is an important factor in determining the extent of the febrile reaction to pyrogens. It seems reasonable, as already stated, to assume that the febrile response to a pyrogen is a reflection of injury. If this assumption is correct the next problem is to determine the site of tissue injury. There is evidence that bacterial pyrogens can cause injury to vascular structures, as was mentioned in the preceding article. Another possible site of injury is the R-E cells themselves. The evidence obtained in this work could be interpreted, however, as indicating that the R-E cells are relatively resistant to injury, and that they serve to protect other, more susceptible cells. Evidence in support of this supposition was obtained in our studies on the effect of R-E blockade on immunity to the Schwartzman phenomenon (3). This phenomenon is advantageous as a method of studying the present problem,

because it provides a defined, observable site of injury in the skin. Some rabbits exhibit natural immunity to the Shwartzman reaction, and in all rabbits an immunity develops after repeated injections of bacterial toxin. It was found that either the natural or the acquired immunity could be abolished by blockade of the R-E system. Following an injection of thorotrast an animal that had previously given no purpuric reaction on test showed severe hemorrhagic necrosis in the skin. These findings can be interpreted as indicating that the blocking substance, by interfering with the cells which normally take up most of the bacterial toxin from the blood, allows the toxin to reach the skin in sufficient quantity to produce the hemorrhagic reaction.

On the basis of the present findings the following hypothesis can be offered regarding the development of tolerance to pyrogens: A series of injections of these causes a change in the functional capacity of the R-E system whereby the ability to remove bacterial toxins from the blood is enhanced. As a result of this increase in ability, other susceptible tissues are protected from injury, and the lessened injury is reflected by a diminution in the febrile response.

#### SUMMARY

In experiments designed to elucidate the mechanism by which tolerance to bacterial pyrogens is developed, the following observations were made:

1. Animals whose febrile reactions to bacterial pyrogens were markedly diminished, as a result of repeated injections, showed increases in response following R-E blockade.

2. Pyrogenic substances disappeared from the circulating blood more rapidly in rabbits rendered pyrogen-tolerant than in normal animals. Lack of specificity was shown by the fact that rabbits previously injected with *Eberthella typhosa* bacterial vaccine were able to remove the pyrogens of *Serratia marcescens* and *Pseudomonas aeruginosa* from their blood more rapidly than normal animals.

3. R-E blockade retarded the speed of disappearance of pyrogens from the circulating blood of animals which had been rendered relatively tolerant by previous injections of these substances.

A possible mechanism for the development of unresponsiveness to bacterial pyrogens is suggested.

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# CYTOCHONDRIA OF NORMAL CELLS, OF TUMOR CELLS, AND OF CELLS WITH VARIOUS INJURIES\*

By EUGENE L. OPIE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 4 AND 5

(Received for publication, March 31, 1947)

The cytoplasm of liver cells consists in large part of vesicular bodies just within the range of microscopic visibility (1). Pathological changes in liver cells and in tumor cells derived from them may bring about such enlargement of these bodies that their structure and relation to one another become readily recognizable. They show a rim which under some circumstances is acidophile and perhaps in large part protein, and under other circumstances is basophile because it contains ribonucleic acid (2, 3), in part at least as nucleoprotein. The greater portion which is within the rim stains faintly, is chiefly lipoid, and consists largely of phospholipids.

All of the bodies with definable rim and clear central space have been designated for convenience, *cytochondria* (cell grains (1)). Certain of them give the reactions that have served to identify mitochondria. These latter are characterized in fixed tissues by their reaction with potassium bichromate which acts as a mordant for several dyes and by their resistance to decolorization when overstained by aniline-acid fuchsin.

It would be unprofitable to review early opinions concerning the granular, fibrillar, or foam-like structure of cytoplasm. Furthermore, it has not seemed desirable to review the extensive controversial literature concerning the relation of mitochondria to secretion, to deposition of fat and glycogen, and to other changes in normal and pathological cells.

## Methods

After fixation in Zenker's fluid and staining by the Giemsa method or by methylene blue and Azur II preceded by phloxin, in accordance with the method of Mallory, the vesicle-like bodies of the cytoplasm are well shown. The usual methods for the demonstration of mitochondria in fixed tissues have been used, and prolonged fixation in Regaud's solution of potassium chromate has been followed by staining with aniline-acid fuchsin or iron hematoxylin. With acid fuchsin alcohol-soluble nigrosin in 1 per cent solution has been a useful counterstain, because it defines cytochondria that do not stain as mitochondria. Staining with iron hematoxylin has been followed by phloxin as a counterstain.

For the purposes of the present study, fixation in a mixture of equal parts of a fifth molar solution of lanthanum acetate (6.85 gm. in 100 cc. water) and 20 per cent formalin has been found to be useful, because it fixes well the ribonucleic acid of the cytoplasm which in many cells of the liver is localized about cytochondria. It has the further advantage that it acts

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\* This study was conducted with the aid of grants from The Jane Coffin Childs Memorial Fund for Medical Research and The Jane Fuller Fund.

promptly as a mordant for the substance that gives to mitochondria a distinctive stain and causes them to be stained by methylene blue used in combination with Azur II. Rose bengal has been substituted for phloxin used by Mallory, because it stains more deeply. Mitochondria identical in size and shape with those demonstrable by other procedures are stained deep blue. Cytochondria that do not stain as mitochondria have a pink color.

Cytochondria become swollen when tissues are subjected to the action of distilled water or of hypotonic solutions and are so much enlarged that they are readily observable. They assume a spherical form and increase in diameter two- or threefold or more. Hypotonic solutions of sodium chloride have a similar effect. It is noteworthy that osmotic swelling of mitochondria has been repeatedly observed (Fauré-Fremiet (4), Anitschkow (5), Lewis and Lewis (6), Lazarow (7)).

Fresh tissue immediately after removal from an animal killed by bleeding from the vessels of the neck has been cut in thin blocks approximately 0.5 mm. in thickness and immersed in distilled water, in hypotonic solutions of sodium chloride, or in solutions of some other substances during periods varying from 5 minutes to 24 hours. The tissue has then been fixed in formalin and lanthanum acetate, Zenker's fluid, absolute alcohol, or Regaud's fluid and embedded in paraffin. For comparison, tissue has been fixed in Regaud's fluid and stained for mitochondria by aniline-acid fuchsin, which stains mitochondria alone, or by iron hematoxylin, which stains both mitochondria and nucleic acid.

#### *Enlargement of Liver Cytochondria in Hypotonic Solutions -*

The effect of water immersion on the cells and cytochondria of the cytoplasm for a time as short as 5 minutes is seen in a narrow zone about the edges of the block of tissue and in the parenchyma surrounding the larger veins which give free access of the fluid to the immediately adjacent tissue. After 1 or 2 hours of immersion, the tissue is changed throughout.

When water enters the liver cells (Fig. 1), they become much swollen and their outlines are accentuated as sharply stained lines. Cytochondria become swollen and spherical and much increased in size. They are seen as isoated acidophile bodies with deeply stained rims and more palely stained or unstained centers. With continued swelling of the cytochondria they are crowded against one another, so that the cytoplasm assumes a vacuolated or foam-like appearance (Fig. 2). The clear centers of the swollen cytochondria seem to form the meshes of a network outlined by the stained rims, now in contact with one another. In liver kept in water from 20 to 24 hours, this appearance may be almost uniform throughout.

Hypotonic solutions have an effect upon the nucleus similar to that upon the cytoplasm. It becomes swollen and in large part loses its nuclear stain. Within the nucleus, round or oval bodies resembling the cytochondria of the cytoplasm but smaller and less sharply defined are recognizable. They have a well stained rim and clearer center and are usually in close contact with one another, but in much swollen nuclei, they may be separated, apparently as discrete bodies.

#### *Osmotic Enlargement of the Cytochondria of the Kidney*

With the Giemsa stain, after fixation in Zenker's fluid, the cytoplasm of the proximal convoluted tubules stains predominantly with the acid dye, and

round, oval, or elongated bodies stained pink form lines perpendicular to the base of the cell. The rim of these bodies is usually more deeply stained than the center and about some of them is a delicate rim of basophile material.

With appropriate stains mitochondria are found crowded together in such abundance that they evidently occupy the greater part of the cytoplasm of the cells of the proximal convoluted tubules and constitute most, at least, of the less well defined bodies seen with the Giemsa stain. They are often much elongated at right angles to the base of the cell and may have a beaded appearance.

When kidney tissue is immersed in distilled water, changes occur more promptly than in the liver (Fig. 3). Mitochondria lose their ability to take the stains that characterize them. The cytoplasm of the swollen cells within 15 minutes may be occupied by bodies with circular outline and vesicular appearance with a diameter much greater than that of the mitochondria. Some of them retain the mitochondrial stain at their periphery. They may be separated from one another as discrete bodies in the swollen cells, but with increased swelling they are crowded together so that the cytoplasm assumes the uniformly vacuolated or foam-like appearance (Fig. 4) seen under similar conditions in the cells of the liver. After immersion in water during 20 hours, some cells with foam-like cytoplasm remain well defined, but in the greater part of the tissue all evidence of structure has disappeared.

#### *Osmotic Enlargement of Cytochondria of the Pancreas*

In the pancreas secretion granules appear as conspicuous round bodies at the apices of the acinar cells, and the basal part of those cells (Zenker, Giemsa, or methylene blue) is occupied by deeply stained basophile material now recognized as ribonucleic acid. Within this basophile material seen in thin sections are a few round or elongated spaces about the size of mitochondria. With mitochondrial stains (Regaud's fluid, aniline-acid fuchsin, or iron hematoxylin) sparsely scattered mitochondria are found in the basal part of the cell. It is noteworthy that the basophile material fills the interstices between the secretion granules in the apices of the cells. After immersion of the tissue in water from 15 to 30 minutes (Fig. 5), two changes occur: basophile substance diminishes or disappears and the vesicular bodies (cytochondria) of the cytoplasm become swollen and finally spherical. In the basal part of the cell, round bodies are closely approximated and much more numerous than the mitochondria demonstrable in this part of the cell by appropriate stains. The secretion granules retain for a time their distinctive stain and resist the penetration of water, but after prolonged immersion (during 20 hours), vacuoles appear uniformly throughout the cell, those replacing the secretion granules being no longer distinguishable from the others.

The cytoplasm of the cells of the pancreatic islets contain minute oval and round mitochondria in great number together with minute bodies of about the

same size and shape with clear center and rim unstained by the mitochondrial stain but defined by the counterstain. They seem to be more resistant to penetration of water than cells of the secreting acini. After immersion in water during 20 hours, mitochondria are stained but in large part vesicular. Cytochondria in the space between them have further increased in size and in places are separated so that they are recognizable as discrete bodies.

#### *Osmotic Enlargement of the Cytochondria of Gland Cells of the Stomach*

The cytoplasm at the apices of the cells forming glands of the stomach contains zymogen granules, but in the basal parts of these cells, it stains deeply with nuclear dyes (Zenker, Giemsa). In this part of the cell in thin sections clear round or oval spaces are surrounded by the basophile material. Here appropriate stains (aniline-acid fuchsin, or iron hematoxylin after fixation in Regaud's fluid) demonstrate the presence of sparsely scattered coarse mitochondria and between them are cytochondria shown by a counterstain but with no mitochondrial stain. When particles of stomach mucosa are immersed in water during 1 or 2 hours, basophile material disappears and mitochondria lose their characteristic stain. Throughout the cytoplasm cytochondria, including mitochondria and zymogen granules, are swollen to form larger spherical bodies recognizable as discrete structures which, when crowded together, give a foam-like appearance to the cytoplasm. Those representing zymogen granules are no longer distinctive and when water imbibition is advanced, are represented by almost uniformly distributed vacuoles.

The cytoplasm of the parietal cells of the gastric mucosa stains by the Giemsa method only with the acid dye, and coarse round or oval mitochondria are demonstrable by the usual methods. In the interstices between mitochondria are bodies of similar form which fail to take the distinctive stain but are defined by the counterstain. When stomach tissue is immersed in water, mitochondria do not lose their peculiar staining character after 1 or 2 hours and do not become swollen, but after immersion during 20 hours, they become vesicular and moderately enlarged.

The cytoplasm of the cells forming the ducts of the liver and pancreas unlike the parenchymatous cells of these organs, contains few and small mitochondria. In the space between these mitochondria are minute bodies of similar size which stain only with the counterstain and have stained rims and clear centers. With immersion in water these cells become swollen and cytochondria are seen as discrete swollen bodies; with greater enlargement they form almost uniformly distributed vacuoles separated by their stained rims. Similar changes are seen in the cells forming the necks of the gastric glands and in those that constitute the collecting tubules of the kidney.

#### *Osmotic Swelling of Tumor Cells*

Osmotic swelling of the cells of hepatomas following the long continued administration of butter yellow causes changes in the cytoplasm of tumor cells



identical with those seen in normal liver cells (Fig. 6). At the edges of pieces of tumor tissue immersed in water changes occur within a few minutes, and after 1 hour these changes may be almost uniform everywhere. Cells are swollen, basophile substance has almost completely disappeared, and distinctive mitochondrial stain is almost wholly lost. Within the swollen cells conspicuous spherical bodies, often well separated from one another and hence recognizable as discrete bodies, occupy the cytoplasm. With increasing swelling of these bodies, all stages are seen in the transition to the foam-like appearance that has been described. Identical changes as the result of water imbibition are seen in a hepatoma caused by the administration of acetylaminoflourene and in sarcomas caused by the introduction of 3,4-benzpyrene in paraffin into the subcutaneous tissue of rats.

#### *Hydropic Swelling of Cytochondria Caused by Dimethylaminoazobenzene*

Following the administration of butter yellow to rats, conspicuous depletion of the basophile substance (ribonucleic acid) of the cytoplasm of liver cells occurs (2) and is often associated with accumulation of fat. The cytochondria in considerable part lose their ability to take up the basic stain and are colored by the acid dye (pink with the Giemsa stain). In sections appropriately stained mitochondria are in general more numerous in cells next to the portal spaces, but cytochondria with no mitochondrial stain are much more abundant than in cells of the normal liver. These are stained by the counterstain and usually occur in groups. As the central vein is approached, mitochondria diminish further and in some cells may be wholly absent, the cytoplasm being occupied in large part by cytochondria that stain only with the counterstain. In places these bodies are enlarged, spherical, and conspicuously vesicular. With greater enlargement, what appear to be vacuoles are formed, and when these are abundant, the cytoplasm has a foam-like appearance. Uniformly vacuolated cells often resemble liver cells with water imbibition, but these vacuoles are often less uniform in size than in tissue exposed to the action of hypotonic solutions. They are recognizable in sections of tissue fixed in lanthanum acetate and formalin, frozen, and stained for fat and evidently contain none.

#### *Hydropic Swelling of Cytochondria Caused by Chloroform*

When the parenchyma about the central veins of the liver is injured by the administration of chloroform (0.25 to 0.3 cc. per 100 gm. of body weight injected subcutaneously with twice its volume of olive oil), necrosis may occur in contact with the vein; it is characterized by loss of nuclei and acidophilia of the cytoplasm. In these cells the cytochondria are in great part swollen and spherical and have sharply defined rims deeply stained by the acid dye. Just outside this area of necrosis, or perhaps in contact with the central vein when necrosis has not occurred, the much swollen, rounded cells have a granular

translucent appearance, because their cytoplasm is occupied by spherical evidently swollen mitochondria which stain faintly with the basic dye. Enlargement of mitochondria may give the cytoplasm a vacuolated or even foam-like appearance, and comparison with frozen sections stained with Sudan IV shows that these vacuoles do not contain fat. Nevertheless, in a broad zone surrounding the swollen granular cells, liver cells contain fat droplets in abundance.

When sections of the liver of rats that have received chloroform as described above are stained for mitochondria (Regaud's fluid, iron hematoxylin, or aniline-acid fuchsin) changes are found in these bodies throughout a large part of the portal unit. Only the liver cells about the portal spaces are like those of the normal liver; they contain well stained mitochondria in great abundance, and bodies of similar size, unstained by the mitochondrial stain, are not readily found; but in the midpart of the radius from portal space to central vein, a large part of the cytoplasm is occupied by bodies that do not take the mitochondrial stain and are stained by the counterstain. In this part of the parenchyma, mitochondria may be rod-shaped or round, and some of the latter shape are apparently losing their ability to take up the mitochondrial stain, so that they stain only at the rim and have a vesicular form. In the swollen cells about the central veins mitochondria are much less abundant and appear as small, scattered, round bodies (Fig. 7). Changes in mitochondria that do not take the mitochondrial stain are best seen when tissues fixed in Regaud's fluid are stained for mitochondria with aniline-acid fuchsin and counterstained with alcoholic nigrosin (Fig. 8). The swollen spherical bodies stained by nigrosin are in some cells well defined as discrete structures and in other cells give a foam-like appearance to the cytoplasm. The changes are identical with those caused by immersion of fresh liver tissue in water.

#### *Accumulation of Fat in the Cytochondria of Liver Cells*

When the mitochondria of liver cells are enlarged by intake of water, their relation to fat deposited in visible stainable droplets becomes evident. To study this relation, accumulation of visible fat in the liver, that is, fatty degeneration, has been produced in the rat by several kinds of injury. Chloroform poisoning causes accumulation of fat chiefly in cells about the central veins or in a zone surrounding necrosis in the same position (8). Withdrawal of food causes the appearance of fat in liver cells (9) about the portal spaces. Removal of one kidney of the rat has been followed in the present study by accumulation of fat in liver cells; and in guinea pigs, fatty degeneration of the liver in the present experiments has followed the administration of diphtheria toxin.

The lesion usually designated as fatty degeneration is an intracellular accumulation of droplets of visible fat following various injuries to parenchymatous

organs. Normal fat metabolism in cells of an organ such as the liver takes place with no deposition of fat droplets within the cells, but injury of cells may bring about accumulation of visible fat. The chemical and physical changes determining the appearance of recognizable droplets of stainable fat have not been defined satisfactorily, but it may be assumed that the deposited fat has failed to undergo the usual transformation into phospholipids and other components of the cytoplasm.

Small pieces of liver, the site of intracellular accumulation of fat, caused by each of the procedures mentioned above, have been immersed in water for 1 hour and after fixation in lanthanum acetate and formalin have been frozen, cut in thin sections, and stained with Sudan IV and hematoxylin (Fig. 9). The cytochondria take up water and become swollen to form spherical bodies of at least twice their usual diameter and especially about the portal spaces their rims are marked by material stained by the basic dye. When fat accumulation is in moderate quantity the contents of scattered cytochondria defined by their basophile rims have taken a deep orange color; whereas those about them may be unstained or stained in lighter shades of yellow. The contents of cytochondria in the same cell may stain with intensity varying from a pale yellow just perceptible to deep orange-yellow. This variation may be explained by assuming that stainable fat gradually accumulates within cytochondria at the expense perhaps of an invisible lipoid content. Cells at the margin of areas of fatty change may contain only a few cytochondria with faint shades of yellow. In the early stages of this fat accumulation, almost all of the fat droplets visible in the cell approximate in size the swollen cytochondria (Fig. 9) and appear as spherical bodies surrounded by a delicate rim of basophile substance; but the larger droplets of more advanced fat change may have no recognizable basophile rim and those of considerable size may very well be formed by the coalescence of smaller droplets.

#### RECAPITULATION AND DISCUSSION

The purpose of this publication is to show that the cytoplasm of the cells that have been studied, including those of several varieties of neoplasm, is in great part constituted by minute bodies which vary much in their chemical composition but have surface properties that make them permeable to water and to other substances in the medium about them. These bodies, which for convenience may be designated cytochondria, in most normal cells are just within the range of microscopic visibility and appear in appropriately stained sections as rounded particles with definable rim and clear central space. They are so much increased in size in certain pathological lesions that further details of their structure become evident (1). When subjected to the action of distilled water or of solutions of certain substances, they enlarge so that they are readily visible and their relation to identifiable components of the cytoplasm

becomes perceptible. Their relation to ribonucleic acid, to water, and to fat indicates that they are concerned with important metabolic changes within the cells and observations that have been recorded here and elsewhere (1) show that they are concerned with a variety of pathological lesions.

Ribonucleic acid is present in or upon the surface of a large part of the cytochondria of the normal liver and is increased when normal liver cells have been transformed into tumor cells (2). Removal of this substance by ribonuclease or other means leaves a body which stains with acid dyes more deeply at the periphery than in the center. The bodies that undergo these changes are in part mitochondria, as characterized by their well known reactions to mordants and to stains. In the parenchymatous cells of the liver the cytoplasm is in great part occupied by bodies with the reactions of mitochondria, but with appropriate methods (fixation in lanthanum acetate and formalin and staining with polychrome methylene blue and rose bengal or fixation in Regaud's fluid and staining with aniline-acid fuchsin and counterstaining with alcohol-soluble nigrosin), bodies of similar form, but unstained by methods for the demonstration of mitochondria, can be found in normal liver cells. When hepatomas are formed from liver cells rendered neoplastic as the result of prolonged feeding with butter yellow, the mitochondria are small and sparsely scattered in the cytoplasm of the tumor cells, while bodies of similar shape giving no mitochondrial reaction but demonstrable by counterstains occupy the greater part of the cytoplasm (1). With a variety of injuries to the cytoplasm of liver cells, well illustrated by poisoning with chloroform, the mitochondria lose in considerable part their peculiar reaction to stains. For convenience all of the bodies that are characterized by a stained rim and clear central space have been designated *cytochondria* (1), the term mitochondria being limited to those with the well known staining reactions. Further its noteworthy that all mitochondria lose their characteristic reactions when fresh tissue is immersed in distilled water but persist as vesicular bodies indistinguishable from other cytochondria.

It has seemed desirable for the purpose of the present study to determine the effect of distilled water and of hypotonic solutions on several tissues including liver, kidney, pancreas, stomach, and some tumors. The results have been nearly uniform. Fluid enters the cell and causes swelling of it. At the same time the fluid causes the cytochondria, including mitochondria, to swell and assume a spherical form. Fluid apparently enters the cell more rapidly than it penetrates into cytochondria so that these are recognizable as discrete, much enlarged, spherical vesicular bodies with stained rim and faintly stained or unstained centers. With further swelling of these bodies, they occupy all of the space within the cell and, crowded against one another, their clear centers seem to form the meshes of a network. The cytoplasm may be said to have a foam-like appearance. The cells of the acini of the

pancreas and of the gastric glands with secretion granules next to the lumina undergo changes similar to those of the liver, and their cytoplasm after 1 or 2 hours of immersion in water appears vacuolated almost uniformly. The swollen bodies that represent the secretion granules are not distinguishable from mitochondria elsewhere in the cytoplasm.

Hydropic swelling of cells with swelling of their mitochondria has been observed in association with butter yellow administration and is well illustrated by the changes that occur in liver cells adjacent to the zones of necrosis about central veins caused by chloroform. The changes are similar to those produced artificially by immersion of fresh tissues in water and reproduce the lesion that has been designated hydropic or vacuolar degeneration. What has long been known as cloudy swelling or granular degeneration is not definable exactly but is represented in part at least by the changes produced by chloroform poisoning. The uncertainty that has existed concerning the relation of the granules of "parenchymatous degeneration" to mitochondria may be in part referable to the associated loss of mitochondrial stain following injury caused by agents such as chloroform or butter yellow. Anitschkow (5) and others have attributed parenchymatous degeneration to swelling of mitochondria.

Some of the changes that occur in mitochondria produce well recognized lesions of which the pathogenesis has been obscure. In hepatomas produced by butter yellow, mitochondria may become swollen and deeply acidophile and, surrounded by a rim of basophile material, may form cellular inclusions resembling those associated with diseases caused by ultramicroscopic viruses (1).

The properties of mitochondria that have been noted serve to define their relation to the fat droplets that accumulate within parenchymatous cells in the presence of injury (fatty degeneration). When liver tissue which has undergone this change, is exposed to the action of distilled water, fat is recognizable within swollen mitochondria marked by rims of basophile material.

#### CONCLUSIONS

Bodies that may be designated *cytochondria* occupy the greater part of the cytoplasm of the normal and tumor cells that have been studied. They are characterized (a) by their behavior as discrete particles with surface properties that cause osmotic changes in the presence of water; (b) by reactions to stains which show that they have a rim surrounding a clearer (lipoid) center; (c) by their varying relation to the basophile substance (ribonucleic acid) of the cytoplasm.

Mitochondria which have characteristic reactions to stains promptly lose their distinctive reactions in the presence of solvents or as the result of pathological changes, becoming apparently indistinguishable from other cytochondria.

Changes that occur in cytochondria give insight into the pathogenesis of

a variety of pathological lesions. Hydropic swelling of cytochondria caused by chloroform, butter yellow, and other agents, representing one variety of parenchymatous degeneration or cloudy swelling, results in changes similar to those following the immersion of fresh tissues in water.

When parenchymatous cells undergo fatty degeneration as the result of injury fat accumulates within cytochondria.

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#### EXPLANATION OF PLATES

These photographs were made by Mr. Joseph B. Haulenbeek.

#### PLATE 4

FIG. 1. To show swelling of cytochondria of cells of liver with formation of discrete spherical vesicular bodies following immersion of the tissue in distilled water during 2 hours. The cytoplasm of one cell (A) has assumed a "foam-like" appearance. Fixation in Regaud's fluid and staining with aniline-acid fuchsin and alcohol-soluble nigrosin.  $\times 1000$ .

FIG. 2. To show advanced swelling of the cytochondria of hepatic cells with "foam-like" appearance of cytoplasm, after immersion in distilled water during 2 hours. Regaud; aniline-acid fuchsin and nigrosin.  $\times 1000$ .

FIG. 3. To show swelling of the cytochondria of cells of the convoluted tubules of kidney with formation of discrete vesicular bodies; immersion in distilled water during  $1\frac{1}{2}$  hours. Regaud; iron hematoxylin and phloxin.  $\times 1000$ .

FIG. 4. To show advanced swelling of cytochondria of the cells of the convoluted tubules of kidney with "foam-like" appearance of cytoplasm. The cytochondrial vacuoles form lines at right angles to the bases of the cells; immersion in distilled water during  $1\frac{1}{2}$  hours. Regaud; iron hematoxylin and phloxin.  $\times 1000$ .

FIG. 5. To show swelling of cytochondria of the cells of a secreting acinus of the pancreas; immersion in distilled water for one-half hour. There is a "foam-like" appearance of the cytoplasm and some basophile substance remains at the bases of the cells and between vesicular bodies elsewhere. Zenker; Giemsa.  $\times 1000$ .

FIG. 6. To show swelling of the cytochondria of cells of a hepatoma produced by administration of butter yellow. The cytochondria can be seen in the much swollen cells as discrete vesicular bodies. Fresh tumor tissue was immersed in tenth molar solution of lanthanum acetate during 6 hours and fixed in formalin. Stained with methylene blue and rose bengal.  $\times 1000$ .



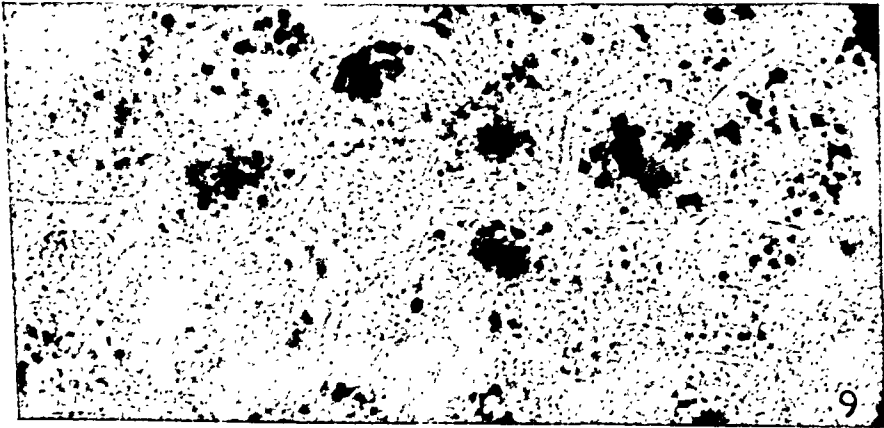
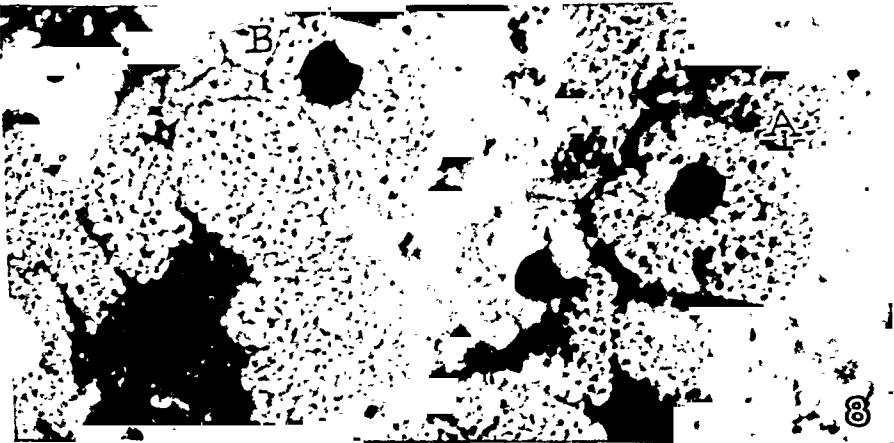
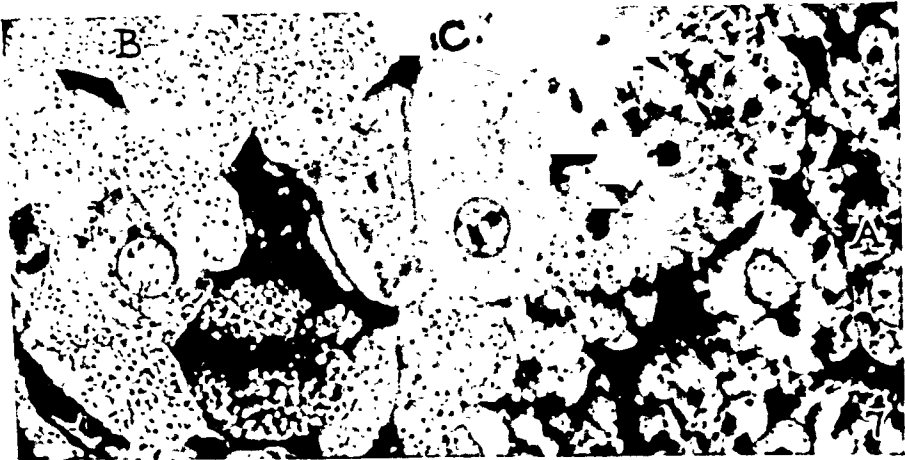
## PLATE 5

FIG. 7. To show swollen cells with mitochondria diminished in number and size in the liver of a rat that had received chloroform (0.25 cc. per 100 gm. of body weight) 24 hours before death by bleeding. Clumps of black material represent the mitochondria in relatively normal liver cells (*A*). In some cells the mitochondria are small and scattered (*B*) and in other cells (*C*) they have almost completely disappeared, but when this is the case, bodies of similar size, not well defined in the photograph because they do not take the mitochondrial stain, replace them. Regaud; iron hematoxylin and phloxin.  $\times 1000$ .

FIG. 8. To show the swollen cells of a rat that received chloroform (0.3 cc. per 100 gm. of body weight) and was killed after 22 hours by bleeding. The sparsely scattered mitochondria are black. Swollen and vesicular cytochondria stained only with the counterstain can be seen in the cells marked (*A*) and others swollen to form vacuoles that give a "foam-like" appearance to the cytoplasm in the cells marked (*B*). Regaud; aniline-acid fuchsin and nigrosin.  $\times 1000$ .

FIG. 9. To show fat and cytochondria in liver cells swollen as the result of immersion of the fresh tissue in distilled water during 1 hour. The animal received no food during 48 hours before death and fat accumulated as droplets in cells about the portal spaces. The tissue after immersion in water was fixed in lanthanum acetate and formalin, sectioned after freezing, and stained with Sudan IV and hematoxylin. The fat appears black. Its inclusion within the basophile rims that define the cytochondria is not visible in the photograph.  $\times 1000$ .







# INHIBITION BY CERTAIN POLYSACCHARIDES OF HEMAGGLUTINATION AND OF MULTIPLICATION OF INFLUENZA VIRUS

BY R. H. GREEN, M.D.,\* AND D. W. WOOLLEY, PH.D.

*(From the Laboratories of The Rockefeller Institute for Medical Research)*

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Antagonism between structurally similar compounds provides a useful tool with which to investigate biological processes. An example of how it has been used as a guide to study virus-host relationships will be described in the present paper.

Examination of many cases of competition between structurally similar substances has led to the conclusion that the inhibitory analogs compete with their related metabolites when the latter function as substrates for a metabolic reaction (1). Now, if one assumes, as Hirst has done (2), that influenza virus, in causing hemagglutination, may be likened to an enzyme attacking a specific substrate in the red cell, then it might be possible to inhibit this activity of the virus by adding a suitably constituted analog of the substrate. Such an inhibition would be analogous to the blocking of the action of succinic dehydrogenase by malonate, a close relative of succinate (3), as well as to numerous other more recent examples (1). Although the postulated substrate which the virus attacks in the red cell is unknown, the observations of Hirst and Hotchkiss (4) that periodate destroys that part of the erythrocyte which reacts with the virus suggested that it is carbohydrate in nature. Therefore, in the work now to be described, a number of simple and complex carbohydrates have been tested for their ability to inhibit agglutination of chicken red blood cells by influenza A virus. The virus-erythrocyte system recommended itself as the simplest case of virus-cell relationship, and it was hoped that any agent found effective in relation to it might find application in an animal host in which multiplication of virus occurs (5).

A number of polysaccharides were found to be capable of inhibiting hemagglutination. Several of the effective agents examined contained large amounts of galacturonic acid, although some were related to other sugars. None of the simple carbohydrates tested showed any activity. Apple pectin, one of the most effective substances, was studied in some detail.

Evidence was obtained that apple pectin exerted an effect on the virus as well as on the erythrocytes. If the working hypothesis were correct, combination between the virus and the pectin would be expected, but reaction of the

\* Fellow in the Medical Sciences of the National Research Council; aided by a grant from the National Foundation for Infantile Paralysis.

pectin with the erythrocytes would not be anticipated. The experimental results suggested that interaction occurred between every possible pair of the three participating substances (virus, polysaccharide, and cell). Therefore, it was of interest to find that from the erythrocyte itself a substance could be extracted which, like the carbohydrates, actively inhibited hemagglutination by the virus.

Because apple pectin was quite able to inhibit virus hemagglutination it was tested for ability to inhibit virus multiplication in embryonated eggs. It was found that pectin was able to do this too. This was true not only when pectin was given before inoculation with the virus but also when it was injected 1 to 2 hours afterwards.

Although the working hypothesis just outlined has led directly to positive experimental results it does not necessarily mean that this hypothesis is the correct one. The actual findings may have been coincidental and quite unrelated to the postulate that influenza virus reacts in an enzymic fashion with a carbohydrate substance in erythrocytes.

The fact that apple pectin inhibits multiplication of virus in embryonated eggs as well as hemagglutination is suggestive but does not prove that the two phenomena are related. However, it is of more than passing interest that alginic acid, a substance closely similar to apple pectin in both physical and chemical properties, showed relatively little activity as an inhibitor of virus hemagglutination and likewise showed little or no potency as an inhibitor of virus multiplication in the egg. On the other hand the substituted nitroacridin 3582, which has been demonstrated to have an inhibitory effect on the growth of influenza B virus in embryonated eggs, exerted no apparent effect on hemagglutination (6). Furthermore, some hemagglutination-inhibiting carbohydrates, *e.g.* citrus pectin, did not reduce multiplication of virus.

### *Materials and Methods*

*Virus Preparations.*—Influenza A virus, PR8 strain, was used exclusively. This strain was obtained as an allantoic fluid preparation from Dr. Frank L. Horsfall, Jr., of the Rockefeller Institute. Allantoic fluids from the third and fourth egg passages made in this laboratory were the source of virus used in the present study. With slight modifications, passages were made and fluids collected and stored according to the directions of Hirst (2).

*Sources and Preparation of Carbohydrates.*—Apple and citrus pectins, gum acacia, gum myrrh, alginic acid, agar, corn starch, galactose, ribose, glucose, and mannose were commercial samples. Preparations of the specific polysaccharide of gum acacia (7), of the blood group A substance, and of cellobiuronic acid were supplied by Dr. W. F. Goebel of the Rockefeller Institute. Galacturonic acid was obtained from Dr. M. A. Stahmann of the University of Wisconsin.

The polyaldehyde of starch was prepared by the method of Jackson and Hudson (8), and the corresponding acid was made by treating an aqueous solution of the aldehyde with an excess of cold sodium hypobromite and dialyzing the reaction mixture to remove excess reagents. The sodium salt of the polysaccharide was then precipitated from the aqueous solution by

adding alcohol. Inositol galactoside tartrate was obtained from soybean lipositol as described by Woolley (9). Neville's method (10) was employed for the preparation of flaxseed mucilage, and the aldobionic acid of rhamnose and galacturonic acid was made from it according to the procedure of Anderson and Crowder (11). An extract from laked chicken RBC was obtained as follows: Stromata were prepared from 150 cc. of washed chicken erythrocytes after hemolysis with distilled water. These ghosts were washed 4 times with saline and then 3 times with water and finally suspended in 200 cc. of water and heated to 100°C. for 5 minutes. The suspension was cooled and a clear solution obtained by high speed centrifugation.

In testing for inhibition of hemagglutination the various materials were dissolved in water, usually in a concentration of 2 per cent, and adjusted to pH 7. Those which did not dissolve in water, e.g. alginic acid and gum myrrh, went into solution on neutralization. Infrequently, it was necessary to remove a small amount of insoluble residue by filtration.

Several methods of preparing solutions of apple pectin for injection into eggs have been tried but that described below was the only one that yielded uniformly good results. Powdered pectin, in small amounts at a time, was gradually sifted onto the surface of water heated to about 100°C. and stirred constantly. The solution was then neutralized with 1 N NaOH, autoclaved (11 pounds for 20 minutes), and when necessary, the pH readjusted to 7 by the addition of a sterile solution of NaOH. This method was time-consuming since about 3 hours' effort was required to obtain 100 cc. of a 5 per cent solution but it produced consistently clear solutions free of masses and insoluble residue. Departure from it often resulted in solutions which contained particulate matter and showed less successful results when tested in eggs.

*Titration of Virus by Agglutination of Chicken RBC.*—Titrations of virus were made by Hirst's procedure (2) except that readings were made by the pattern test reported by Salk (12). Serial twofold dilutions of allantoic fluid were made in saline,<sup>1</sup> using 0.5 cc. amounts. To each tube was then added 0.25 cc. of a 1 per cent suspension of washed RBC from individual, adult chickens. Results were read after the tubes had stood in a vertical position at room temperature (23–26°C.) for 2 hours. The highest dilution at which complete agglutination occurred was taken as the end-point and this amount of virus was arbitrarily considered one agglutinating unit.

The following criteria, identical in most respects with those described by Salk (12), were used in reading the tests: (1) A pattern considered characteristic of complete agglutination was designated as c (complete). This pattern consisted of a uniformly thin layer of cells completely covering the concave bottom of the tube. It was easily seen as a salmon-pink layer of cells when the tube was viewed either from the side or from the bottom. (2) A pattern considered characteristic of the absence of agglutination was designated as 0. Here, the cells did not stick to the margins of the bottom but slid down to the center forming a small, compact, dark red mass with sharply defined, smooth edges. When tubes containing such patterns were tilted horizontally the cells ran smoothly down the bottom and onto the side of the tube, much in the manner of a drop of ink running down an inclined plane surface. (3) A pattern considered intermediate between c and 0 was designated as p (partial). This type apparently consisted of large clumps of cells which had settled to the center of the bottom of the tube but invariably occupied a larger area than the patterns designated 0; had ragged edges, and did not run smoothly on horizontal tilting. Furthermore, such patterns frequently showed small areas of a thin layer of cells around their margins and a thin rim of cells still attached to the upper periphery of the concave bottom of the tube. (4) A pattern considered as closely approaching that described as 0 was designated t (trace). On casual inspection, this pattern could not be differentiated easily from 0, especially when viewed from

<sup>1</sup> Here, as elsewhere, saline means 0.85 per cent NaCl.

the side of the tube. It too consisted of a small, compact, dark red mass, occupying the center of the bottom of the tube. However, on closer inspection it was found that such patterns were slightly larger than the 0 type. In addition, the edges were somewhat granular and on horizontal tilting, when movement occurred, the cells tended to move as a single piece, rather than run smoothly.

*Titration of Virus by Infectivity for Embryonated Eggs.*—Serial tenfold dilutions of allantoic fluids to be tested were made in phosphate buffer<sup>2</sup> and 0.05 cc. amounts of appropriate dilutions injected into the allantoic sacs of each of 2 to 5 embryonated eggs on the 10th day of incubation. Incubation at 37.5°C. was continued for 48 hours, followed by chilling at 4°C. for 18 to 20 hours. Allantoic fluid was then removed from each egg, and tested for its ability to produce hemagglutination.

### *Inhibition of Virus Hemagglutination by Various Carbohydrates*

Serial twofold dilutions of 2 per cent solutions of the substances tested were made in saline in 0.25 cc. amounts. To each tube was then added 0.25 cc. of a 1 per cent suspension of chicken RBC. This was followed by the addition of 0.25 cc. of virus-containing allantoic fluid so diluted in saline that each 0.25 cc. contained approximately 4 hemagglutinating units of virus (a 1-160 dilution of allantoic fluid). Positive controls consisting of only virus and cells and negative controls of only saline and cells were included and the results were read after 2 hours at room temperature.

The data presented in Table I show that several of the materials tested prevented the formation by influenza A virus of typical patterns of hemagglutination. Those showing a considerable effect were flaxseed mucilage, citrus pectin, apple pectin, blood group A substance, gum acacia, gum myrrh, and the extract of chicken RBC. Many of the substances showing inhibitory activity were polysaccharides rich in galacturonic acid. However, polygalacturonides were not unique in this respect because gum acacia and concentrates of the blood group A substance, both of which do not contain galacturonic acid, were effective. Alginic acid, a polysaccharide largely composed of mannuronic acid units, was practically inactive. Furthermore, complex carbohydrates such as starch and the polyaldehyde or polyacid prepared from it by oxidation caused no inhibition of hemagglutination. None of the simple carbohydrates, such as galactose, galacturonic acid, the aldobionic acid from flaxseed mucilage, etc., exhibited any activity. The chemical composition and homogeneity of such materials as the pectins, plant mucilages, and gums are not sufficiently established to justify exact conclusions as to the nature of the configuration required for inhibitory action.

### *Effect of Various Carbohydrates on Chicken RBC in the Absence of Virus*

Because a wide variety of substances are known to cause hemagglutination (13, 14) experiments were performed to determine whether the materials studied had any effect on RBC in the absence of virus. Tests identical with those

<sup>2</sup> Except where otherwise indicated, phosphate buffer means 0.1 M sodium phosphate buffer at pH 7.

described in the preceding section, except that virus was replaced by saline, were set up. For facility in comparison these data also are presented in Table I. Several of the preparations tested in this manner caused some hemagglutination. Most of those capable of inhibiting virus hemagglutination did

TABLE I  
*Effect of Various Substances on Hemagglutination of Chicken RBC in the Presence of Influenza A Virus and Without Virus*

Test substance	With influenza virus										Without influenza virus									
	Final concentration of test substance, $\gamma/cc.$										Final concentration of test substance, $\gamma/cc.$									
	6666	3333	1666	833	416	208	104	52	26	13	6666	3333	1666	833	416	208	104	52	26	13
Flaxseed mucilage		c	c	p	p	t	p	p	p	p		c	p	p	t	t	t	0	0	0
Galacturonic acid	c	c	c	c	c	c	c	c	c	c	0	0	0	0	0	0	0	0	0	0
Cellobiuronic acid	c	c	c	c	c	c	c	c	c	c	0	0	0	0	0	0	0	0	0	0
Inositol galactoside tartrate	c	c	c	c	c	c	c	c	c	c	t	t	t	0	0	0	0	0	0	0
Citrus pectin	p	t	t	t	t	t	p	c	p	p	p	0	0	0	0	0	0	0	0	0
Apple pectin	p	p	p	t	t	t	p	p	p	p	p	0	0	0	0	0	0	0	0	0
Blood group A substance	p	p	t	p	p	t	t	p	p	p	0	0	0	0	0	0	0	0	0	0
Galactose	c	c	c	c	c	c	c	c	c	c	0	0	0	0	0	0	0	0	0	0
Flaxseed mucilage aldo-bionic acid	c	c	c	c	c	c	c	c	c	c	c	p	0	0	0	0	0	0	0	0
Glucose	c	c	c	c	c	c	c	c	c	c	0	0	0	0	0	0	0	0	0	0
Mannose	c	c	c	c	c	c	c	c	c	c	0	0	0	0	0	0	0	0	0	0
Alginate acid	p	p	p	p	c	c	c	c	c	c	t	t	0	0	0	0	0	0	0	0
Soluble starch*	c	c	c	c	c	c	c	c	c	c	0	0	0	0	0	0	0	0	0	0
"Starch polyacid"	c	c	c	c	c	c	c	c	c	c	0	0	0	0	0	0	0	0	0	0
Gum acacia	c	c	c	p	p	p	t	p	p	p	c	p	0	0	0	0	0	0	0	0
RBC extract				p	p	t	p	p	p	p				0	0	0	0	0	0	0
"Starch polyaldehyde"	c	c	c	c	c	c	c	c	c	c	0	0	0	0	0	0	0	0	0	0
Gum myrrh*	p	p	p	p	p	p	p	p	p	p	0	0	0	0	0	0	0	0	0	0
Gum acacia specific polysaccharide	c	c	c	c	c	c	c	c	c	c	0	0	0	0	0	0	0	0	0	0
Ribose	c	c	c	c	c	c	c	c	c	c	c	c	p	0	0	0	0	0	0	0
Agar agar																		p	0	0
Normal rabbit serum	0	t	t	t	t	t	p	p			0	0	0	0	0	0	0	0		

\* Weight includes insoluble residue which was discarded.

so. However, a significant effect occurred only at concentrations higher than those at which there was inhibitory activity. The meaning of this was not clear, especially when it was found that some substances by themselves caused hemagglutination but showed no inhibitory action.

Additional evidence that the polysaccharides affected the RBC was obtained by showing that cells treated with a suitable concentration of apple pectin and then washed with saline did not behave in the same fashion, when mixed with influenza virus, as did untreated cells. Thus, 0.25 cc. of a 1 per cent suspension of cells mixed with 0.25 cc. of apple pectin (3 mg. per cc.) plus 0.25 cc. of saline showed no agglutination after 2 hours at 25°C. The tubes were then centrifuged lightly and the cells washed 5 times with saline and finally resuspended in

0.75 cc. saline. After the cells had sedimented 0.25 cc. of the supernatant was removed and replaced by 0.25 cc. of saline containing 4 agglutinating units of virus; the cells were resuspended and allowed to settle in the usual way. Readings then showed that only partial hemagglutination (readings of t or p) had occurred. However, when these cells were again washed several times and another 4 units of virus were added complete agglutination occurred. Furthermore, cells suspended in a hemagglutination-inhibiting concentration of apple pectin and treated with virus before washing behaved in a similar manner.

### *Effect of Apple Pectin on Virus*

In order to determine whether apple pectin had any effect on the virus itself mixtures of the two were made and tested for infectivity in embryonated eggs.

1.8 cc. of a solution of apple pectin (10 mg. per cc.) in 0.08 M sodium phosphate buffer at pH 7 were mixed with 0.2 cc. of virus-containing allantoic fluid. After the mixtures had stood at 23–26°C. for 45 minutes they were titrated for infectivity in embryonated eggs as previously described. Control mixtures in which phosphate buffer was substituted for apple pectin were examined simultaneously. In one experiment alginic acid, in the same concentration a pectin, was used as a further control. The  $ID_{50}$  titers of virus treated with pectin in this manner were approximately  $10^{-7}$  whereas those treated with alginic acid and phosphate buffers alone were approximately  $10^{-8}$ .

The results of four such experiments showed that the titer of virus mixed with pectin was reduced by about 1 log.

### *Effect of Varying the Order of Mixing Apple Pectin, Cells, and Virus*

The inhibition of virus hemagglutination effected by apple pectin was demonstrable when the reactants were mixed in the order previously described; i.e., apple pectin, plus cells, plus virus. Moreover, when pectin and virus were mixed together and cells added to the mixture a similar inhibitory effect was observed. However, when virus and cells were first mixed and pectin was then added no inhibition was obtained. This finding indicated that the inhibition of hemagglutination was not due to a change in viscosity or some other physical property of the system, brought about by the pectin.

### *Difference between Inhibition of Hemagglutination Caused by Apple Pectin and That Caused by Normal Rabbit Serum*

The fact that normal animal and human sera inhibit hemagglutination by influenza virus has been demonstrated repeatedly (2, 15, 16). The data in Table I illustrate this again. However, in contrast to the importance of the order of mixing for the inhibitory action of apple pectin, normal rabbit serum (heated at 56°C. for 30 minutes) caused inhibition of hemagglutination regardless of the order. Thus normal rabbit serum, when added after virus and cells were mixed, prevented hemagglutination, whereas apple pectin did not.



*Inhibition by Apple Pectin of Virus Multiplication in Embryonated Eggs*

1. *When Pectin Was Injected  $\frac{1}{2}$  Hour before Virus.*—One cc. of a 5 per cent solution of apple pectin was injected into the allantoic sac of each of a number of embryonated eggs on the 10th day of incubation. One-half hour later approximately 100 ID<sub>50</sub> of influenza A virus, in a volume of 0.05 cc., were introduced into the allantoic sac. After incubation at 37.5°C. for 48 hours followed by chilling at 4°C. for 18 to 20 hours, the allantoic fluids were collected and titrated individually for the presence of virus by hemagglutination, and in some instances by infectivity for embryonated eggs. Eggs which had received virus alone or virus plus 1 cc. amounts of saline or a 5 per cent solution of alginic acid served as suitable controls.

Results illustrative of the best that were obtained are shown in Table II. As judged by the hemagglutination tests there had been considerable inhibition of virus multiplication in all of the pectin-treated eggs. In order to be sure that there was, actually, a reduction in the amount of virus, and that the presence of pectin in these allantoic fluids was not interfering with hemagglutination several of them were tested for infectivity. The results confirmed the hemagglutination findings and furthermore, showed that in the fluids tested no virus was detectable by this method. By subsequently adding known amounts of virus to these fluids it was shown that the presence of residual pectin in them did not significantly interfere with the detection of virus by means of hemagglutination.

Four experiments like the one depicted in Table II revealed that of a total of 37 eggs thus treated with pectin only 5 were positive for hemagglutination. All controls were positive. Alginic acid was chosen as a control because of its similarity in physical and chemical properties to apple pectin and because it showed only a slight ability to inhibit virus hemagglutination.

Furthermore, when judged by the results of hemagglutination tests, 25 mg. of apple pectin per egg afforded almost as much protection against 100 ID<sub>50</sub> of virus as did 50 mg. A decrease in virus multiplication was observed when 10,000 and even when 1,000,000 ID<sub>50</sub> of virus were inoculated after the injection of 50 mg. of pectin. For example, of 5 eggs given 10,000 ID<sub>50</sub> of virus all 5 were negative and of 5 eggs given 1,000,000 ID<sub>50</sub> 2 were negative.

Pectin injected into the yolk sac in 50 mg. amounts either  $\frac{1}{2}$  hour or 24 hours before inoculation of virus into the allantoic sac was not effective in inhibiting the multiplication of virus.

2. *When Pectin Was Injected 1 Hour after Virus.*—Embryonated eggs on the 10th day of incubation were inoculated with approximately 100 ID<sub>50</sub> of virus and 1 hour later 1.0 cc. of a 5 per cent solution of apple pectin was injected into the allantoic sac of each. Appropriate controls were inoculated with virus alone and with virus followed by 1.0 cc. amounts of saline or 5 per cent solutions of alginic acid. The eggs were incubated and fluids harvested as previously described. Results were determined by hemagglutination and, in some instances, by infectivity also. Of a total of 25 eggs so treated the allantoic fluids of 15 or 60 per cent have not contained sufficient virus to produce hemagglutination.

TABLE II

*Effect of Various Substances, When Injected 1/2 Hour before Virus, on the Multiplication of Influenza A Virus in Embryonated Eggs*

Experiment No.	Substance injected	Amount		Egg No.	Dilutions of allantoic fluid										
					Hemagglutinin titers						Infectivity titers				
					10	20	40	80	160	320	640	10	10 <sup>2</sup>	10 <sup>3</sup>	10 <sup>4</sup>
1	Apple pectin	cc.	mg.	1	0	0	0	0	0	0	0	0	0		0
				2	0	0	0	0	0	0	0	0		0	
				3	0	0	0	0	0	0	0	0		0	
				4	0	0	0	0	0	0	0	0		0	
				5	0	0	0	0				0	0	0	
				6	0	0	0	0	0	0	0	0		0	
				7	0	0						0	0	0	
				8	0	0	0					0		0	
				9	0	0	0	0	0	0	0	0		0	
				10	0	0	0	0	0	0	0				
				11	0	0	0	0	0	0		0	0	0	
				12	0	0	0	0	0	0	0	0		0	
				13	0	0	0	0	0	0	0	0	0	0	
				14	0	0	0	0	0	0	0				
				15	0	0	0	0	0	0	0				
				16	0	0	0	0	0	0	0				
				17	0	0	0	0	0	0	0				
				18	p	0	0	0	0	0		0	0	0	
				19	0	0	0	0	0	0	0				
				20	0	0	0	0	0	0	0				
1	Sodium chloride	1.0	8.5	21	c	c	c	c	c	c	c				0
				22	c	c	c	c	c	c	c				
				23	p	p	p	c	c	c	c				
				24	c	c	c	c	c	c	c				+
				25	c	c	c	c	c	c	c				+
				26	c	c	c	c	c	c	c				+
				27	c	c	c	c	c	c	c				
				28	c	c	c	c	c	c	c				
1	None			29	c	c	c	c	c	c	c				+
				30	c	c	c	c	c	c	c				+
				31	c	c	c	c	c	c	c				+
				32	c	c	c	c	c	c	c				
				33	c	c	c	c	c	c	c				
				34	c	c	c	c	c	c	c				
5	Alginic acid	1.0	50	35	c	c	c	c	c	c	c				
				36	c	c	c	c	c	c	c				
				37	c	c	c	c	c	c	c				
				38	c	c	c	c	c	c	c				
				39	p	c	c	c	c	c	c				

Results of a typical experiment are shown in Table III. It is evident that pectin given 1 hour after inoculation of virus exerted a significant inhibition of virus multiplication whereas alginic acid did not. The infectivity titers, however, showed that, in most instances, some multiplication had occurred. A less marked, but still appreciable, effect was obtained when the interval between injection of virus and pectin was increased to 2 hours.

TABLE III

*Effect of Apple Pectin and Alginic Acid, When Injected 1 Hour After Virus, on the Multiplication of Influenza A Virus in Embryonated Eggs*

Substance injected	Egg No.	Dilutions of allantoic fluid													
		Hemagglutinin titers									Infectivity titers				
		10	20	40	80	160	320	640	1280	2560	0	10 <sup>+</sup>	10 <sup>+</sup>	10 <sup>+</sup>	10 <sup>+</sup>
Apple pectin	1	0	0	0	0	0	0	0	0	0	+	+	±*		
	2	0	0	0	0	0	0	0	0	0	+	+	0		
	3	0	0	0	0	0	0	0	0	0	+	0	0		
	4	p	p	p	0	0	0	0	0	0					
	5	0	0	0	0	0	0	0	0	0	0	0	0		
	6	c	p	0	0	0	0	0	0	0		+	+	+	±
	7	0	0	0	0	0	0	0	0	0	+	0	0		
	8	0	0	0	0	0	0	0	0	0					
Alginic acid	9	c	c	c	c	c	c	c	c	p			+	+	+
	10	c	c	c	c	c	c	c	p	p			+	+	+
	11	c	c	c	c	c	c	c	c	p			+	+	0
None	12	c	c	c	c	c	c	c	c	c				+	+
	13	c	c	c	c	c	c	c	c	c				+	+

\* Indicates that, at this dilution, approximately half the eggs used for testing were positive.

*Effect of Other Hemagglutination-Inhibiting Substances on Multiplication of Virus in Embryonated Eggs*

A number of substances showing hemagglutination-inhibiting effect were tested, in the manner described above, for the ability to inhibit multiplication of influenza virus in embryonated eggs.

Citrus pectin, in 50 mg. amounts, flaxseed mucilage, in 10 mg. amounts, and undiluted normal rabbit serum in 1 cc. amounts, did not inhibit the multiplication of virus. Gum acacia, in 50 mg. amounts, did show a significant inhibitory effect when injected one-half hour before virus but showed no effect when injected one hour after the virus.

*Toxicity of Apple Pectin for Embryonated Eggs*

Apple pectin in 50 mg. amounts (1 cc. of 5 per cent solution) was relatively non-toxic when injected into the allantoic sacs of embryonated eggs on the 10th day of incubation. Thus, in a preliminary experiment all 3 eggs of a group so treated survived to the time of hatching. Subsequent experiments in which virus and 50 mg. amounts of pectin were injected into the allantoic sac showed that of 196 eggs 28, or about 14 per cent, died within the 48 hour incubation period.

## SUMMARY

The complex carbohydrates apple pectin, citrus pectin, flaxseed mucilage, blood group A substance, gum acacia, and gum myrrh as well as an extract of RBC, when examined in a pattern test, were shown to inhibit the agglutination of chicken RBC by influenza A virus. A number of other simple and complex carbohydrates showed no inhibitory effect. The hemagglutination-inhibiting action of apple pectin was examined in some detail and evidence was adduced to show that it affected both virus and red cell. Apple pectin was also found to inhibit the multiplication of influenza A virus in embryonated eggs.

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# THE NATURE OF NON-SPECIFIC INHIBITION OF VIRUS HEMAGGLUTINATION\*

BY WILLIAM F. FRIEDEWALD, M.D., EDWARD S. MILLER, M.D., AND  
L. ROSS WHATLEY

(From the Department of Bacteriology and Immunology, Emory University  
School of Medicine, Atlanta)

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The agglutination of red blood cells by certain viruses is the function of a specific receptor substance associated with the cells, which combines with the virus (1). This reaction has provided *in vitro* methods for detecting these viruses and also their antibodies, since the latter specifically inhibit the agglutination reaction. It has been observed, however, that non-specific inhibition of virus hemagglutination may be caused by a variety of materials including normal sera (2), extracts of animal tissues (3), normal allantoic fluid (4, 5), and human tears (6). Of interest in this respect is the finding that hemagglutination with PVM virus in mouse lung extracts is not manifest unless the extracts are heated to 70°C. (7), a procedure which apparently releases the virus from a non-specific inhibitory factor (8).

In the course of studies on influenza virus antibodies in human tissues, a factor other than specific antibody was encountered which prevented hemagglutination with influenza or mumps viruses. Further investigation of this reaction has provided evidence that the virus receptor substance which has been released from cells may be responsible for the inhibition reaction. The experiments are reported in the present paper.

## Methods

*Preparation of Virus Suspensions.*—The PR8 strain of influenza A virus and the Lee strain of influenza B virus were used in these experiments. Suspensions of the viruses were prepared by inoculating egg-passaged virus into the allantoic sac of 11 day old chick embryos. After 48 hours at 37°C., the eggs were chilled overnight at 4°C. and the blood-free allantoic fluid removed. The virus suspensions were then cleared by centrifugation at 3,000 R.P.M. for 15 minutes.

The strain of mumps virus used was obtained through the courtesy of Dr. J. F. Enders as amniotic fluid of the 36th passage in embryonated eggs. The virus was inoculated into the amniotic sac of embryonated eggs on the 7th or 8th day of incubation. After further incubation at 37°C. for 4 days, the amniotic fluid was removed and cleared by low speed centrifugation.

All virus suspensions were stored at -76°C.

*Hemagglutination Tests.*—Titration of the hemagglutinin in influenza and mumps virus suspensions was done according to the method of Salk (9). In the agglutination inhibition

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test 0.25 cc. of dilutions of serum or tissue extract was mixed with an equal volume of virus suspension containing a known concentration of hemagglutinin. After 1 hour at room temperature 0.5 cc. of a 0.25 per cent suspension of washed chicken red blood cells was added with a pipetting machine. The final concentration of agglutinin in the system was 2 units, unless otherwise indicated. The results were read after 1½ hours at room temperature. The titers were recorded as the reciprocal of the highest dilution producing complete inhibition.

#### EXPERIMENTAL

*Agglutination Inhibition Tests with Human Serum and Tissue Extracts.*—Human autopsies were first used as a source of material for studying agglutination inhibition titers of serum and tissue extracts.

Serum and tissues were obtained from a total of ten autopsies, which were performed from 1 to 7 hours after death. Only tissues which showed no gross or microscopic evidence of disease were included in this study. Ten per cent saline extracts of the tissues were prepared by grinding weighed portions with alundum and suspending in saline. The extracts were cleared by centrifugation first at 3,000 R.P.M. for 15 minutes and again at 12,000 R.P.M. for 15 minutes. Blood was obtained from the heart, and the serum was separated after clot retraction. Serial dilutions of the extracts and serum were tested for capacity to inhibit 2 units of the PR8 and Lee strains of influenza virus and the mumps virus, as described under Methods.

Table I summarizes the results obtained with tissue extracts and serum from three autopsies, which are representative of the findings as a whole. Patient 1 was a normal full term infant who died during delivery. The autopsy was performed 2 hours after death. The second patient was a 4 year old boy who died shortly after arrival at the hospital. The cause of death was not determined. The only findings at autopsy, which was performed 1 hour after death, were cardiac hypertrophy and dilatation, some pulmonary edema, subendocardial hemorrhages, and submucosal hemorrhages of the stomach. Cultures of the lung and blood showed no growth and a nasopharyngeal culture was negative for *C. diphtheriae*. Patient 3 was a 56 year old man who died suddenly following myocardial infarction. Autopsy was done 4 hours after death.

In general, it will be seen (Table I) that inhibition of agglutination with the influenza and mumps viruses was obtained in high titer with all of the materials tested. Extracts of human spleen were also found to cause inhibition in high titer (not shown in the table). Of particular interest was the finding that the titers of the organ extracts were often higher than the serum titers. Previous work on comparative antibody titers of serum and tissue extracts against the typhoid bacillus (10) and the papilloma virus (11) has revealed a much higher titer of specific antibodies in the serum than in the tissue extracts. It was suspected, therefore, that some factor other than specific antibody was responsible for the inhibition of the agglutination reaction.

*Effect of Heat on the Inhibitory Factor in Human Serum and Tissue Extracts.*—Tests were next done to determine the effect of heat on the capacity of human serum and tissue extracts to inhibit virus hemagglutination.

Aliquots of serum diluted 1:10 and 10 per cent extracts of human tissues were heated at 56°, 65°, and 75°C. for 30 minutes. The precipitates which formed were removed by centrifugation at 3,000 R.P.M. for 15 minutes and again at 12,000 R.P.M. for 15 minutes. Serial dilutions of unheated and heated serum and tissue extracts were then tested with 2 units of PR8, Lee, and mumps viruses.

Table II shows the results of an experiment with material from a 2 months old infant, which had unusually high inhibition titers. It will be seen that

TABLE I  
*Agglutination Inhibition Titers of Human Serum and Tissue Extracts*

Patient No.*	Test virus†	Agglutination inhibition titers‡				
		Serum	Saline extracts			
			Lung	Liver	Kidney	Muscle
1	PR8	640	640	2560	640	40
	Lee	640	1280	640	1280	320
	Mumps	1280	1280	640	1280	320
2	PR8	1280	2560	10,240	2560	
	Lee	320	1280	2560	640	
	Mumps	320	1280	640	640	
3	PR8	1280	640	640	1280	80
	Lee	320	320	640	160	80
	Mumps	1280	1280	1280	2560	320

\* See text for description of cases and autopsy findings.

† Two hemagglutinating units of virus.

‡ Titers are expressed as reciprocal of highest dilution causing complete inhibition of hemagglutination. The dilution factor of the extracts is calculated in terms of wet weight of the tissue.

heating up to 75°C. for 30 minutes had no significant effect on the inhibitory substance in serum. In general, however, the titers of the tissue extracts were reduced by heating to 75°C.

It is obviously difficult from experiments with human serum and tissue extracts to decide whether we are measuring virus antibodies or a non-specific factor, since most human sera contain influenza and possibly mumps virus antibodies. It is well known, however, that the generality of antibodies are destroyed by heating at 75°C. Furthermore, some of the serum and tissue extracts with high inhibition titers had no demonstrable neutralizing antibodies. It seemed reasonable, therefore, to conclude that at least a part of the inhibition titers observed with serum and extracts of human tissues were due to some factor causing non-specific inhibition of the agglutination reaction.

It is recognized that the preceding findings might be criticized since they

TABLE II  
*Effect of Heat on Inhibitory Substance*

Test virus	Material*	Agglutination inhibition titer			
		Not heated	Heated 30 min.†		
			56°C.	65°C.	75°C.
PR8	Serum	5120	5120	5120	5120
	Lung	10,240	20,480	5120	2560
	Liver	40,000	2560	640	160
	Kidney	20,480	20,480	10,240	10,240
	Muscle	2560	1280	2560	320
Lee	Serum	2560	5120	2560	2560
	Lung	20,480	20,480	5120	5120
	Liver	10,240	320	160	<80
	Kidney	20,480	2560	1280	640
	Muscle	1280	160	80	<80
Mumps	Serum	1280	640	640	640
	Lung	10,240	5120	1280	640
	Liver	2560	160	80	<80
	Kidney	5120	320	80	<80
	Muscle	1280	80	80	80

\* Tissues obtained from 2 month old infant with congenital heart disease, 1 hour after death.

† Serum and extracts 1:10 in saline.

TABLE III  
*Inhibition Titers of Normal Rabbit Serum and Tissue Extracts*

Normal rabbit	Agglutination inhibition titer			
	Not heated		56°C. 30 min.	
	Test virus		Test virus	
	PR8	Lec	PR8	Lec
Serum.....	160	160	160	160
Red cell extract.....	40	40	<40	<40
Lung extract.....	20,480	20,480	10,240	20,480
Liver ".....	2560	10,240	40	1280
Kidney ".....	20,480	10,240	2560	2560
Muscle ".....	320	320	<40	<40

were obtained with autopsy material. Comparable experiments were undertaken, therefore, with normal rabbit and guinea pig tissues. Table III summarizes the results obtained in a typical experiment with serum and tissue extracts from a normal rabbit. The findings are similar to those observed with



human tissues in that extracts of the tissues had higher inhibition titers than the serum and heating at 56°C. had no detectable effect on the serum or lung extract titers but reduced the titers of the liver, kidney, and muscle extracts. Similar findings were observed with guinea pig serum and tissue extracts. Hirst has already reported that extracts of ferret lung cause marked inhibition of influenza virus agglutination (3). Obviously, specific antibodies cannot be responsible for the inhibition of virus hemagglutination produced by serum and tissue extracts of normal animals.

*Effect of Hemoglobin on Inhibition Titers of Organ Extracts.*—The extracts tested in the preceding experiments contained considerable amounts of hemoglobin due to extraction of the red blood cells present in the tissues. Attempts were therefore made to obtain extracts with as little hemoglobin as possible. For this purpose, lungs were removed from normal rabbits and guinea pigs. Immediately after removal, one-half of the lung was thoroughly perfused with physiological saline through the pulmonary artery, while the other half was not perfused. Saline extracts of the perfused and non-perfused lungs were then prepared and the capacity of each to inhibit influenza virus hemagglutination was determined, as previously described. Although extracts of the perfused lungs contained no visible hemoglobin, they still caused inhibition. The virus inhibition titers, however, when compared with extracts of non-perfused lung taken from the same animal, were usually reduced two- to fourfold.

The decrease in inhibition titers of organ extracts observed in these experiments indicated that both tissue cells and red blood cells yielded the inhibitory substance. The latter possibility was tested further in the next experiments.

#### *Inhibitory Substance in Red Cell Extracts.*—

Human, chicken, sheep, and rabbit red blood cells were obtained in citrate and washed three times in physiological saline. The hemagglutinin titer of PR8, Lee, and mumps virus preparations was determined with each of the red cell suspensions (0.25 per cent) in the usual way. The red cells were then extracted in saline in a Waring blender (1:20 by volume of packed cells) and centrifuged two times at 3,000 R.P.M. for 15 minutes. Serial dilutions of the supernatant fluids were tested for capacity to inhibit agglutination of chicken red cells as already described, using 2 units of PR8, Lee, and mumps viruses.

Table IV illustrates the results obtained in numerous tests with human and animal red blood cells. The findings clearly indicate a correlation between the capacity of the red cells to agglutinate with these viruses and the inhibition titers produced by extracts of the red cells. Human and chicken red cells agglutinated readily with these viruses and extracts of the cells caused inhibition of agglutination in high titer. Rabbit red cells, on the other hand, agglutinated weakly with the viruses and their extracts caused little or no inhibition (see also Table III). Sheep red cells varied in their capacity to agglutinate with mumps and influenza virus and also in their yield of the inhibitory substance.

In the experiment with sheep cells recorded in Table IV, agglutination was observed only with the mumps virus, and extracts of the cells inhibited hemagglutination only with mumps virus and not with the PR8 or Lee influenza virus.

TABLE IV

*Comparison of Virus Receptor Substance and Inhibitory Substance of Red Blood Cells*

Red blood cells	Agglutination titer with			Inhibition titer* red cell extracts		
	Influenza virus		Mumps virus	Test virus		
	PR8	Lee		PR8	Lee	Mumps
Human.....	256	1024	1024	2560	1280	2560
Chicken.....	512	2048	1024	5120	1280	5120
Sheep.....	<32	<32	256	<80	<80	2560
Rabbit.....	<32	<32	<32	<80	<80	<80

\* Expressed as highest dilution of extracts causing complete inhibition of chicken red cell agglutination with 2 units of test virus (see text).

TABLE V

*Effect of Removal of Receptor Substance from Red Blood Cells*

Chicken red cells treated with*	Agglutination of red cells†	Agglutination inhibition titers of red cell extracts		
		Test virus		
		PR8	Lee	Mumps
Saline.....	+	1280	1280	640
Normal allantoic fluid.....	+	1280	1280	640
PR8 allantoic fluid.....	0	<160	<160	<160
Lee allantoic fluid.....	0	<160	<160	<160

\* 37°C. for 17 hours (see text).

† With PR8, Lee, and mumps viruses.

Further experiments revealed that extracts of human and chicken red cells did not neutralize influenza virus in mice. Furthermore the extracts failed to fix complement in mixtures with influenza and mumps viruses.

Group O human red cells were ordinarily used in these experiments. However, tests with group A and with group B red cells showed no significant difference in the yield of the virus inhibitory substance. Furthermore, purified group A and B substances<sup>1</sup> caused no inhibition of red cell agglutination with PR8, Lee, or mumps viruses. Extracts of Rh-positive and Rh-negative cells

<sup>1</sup> We are indebted to Sharp and Dohme, Incorporated, Glenolden, Pennsylvania, for the supply of blood group specific substances A and B.

both caused inhibition of virus agglutination. It would appear, therefore, that the blood group substances A and B and the Rh factor are distinct from the substance obtained from human red cells which is responsible for the inhibition of virus agglutination.

Experiments on the effect of heat on the virus inhibitory substance in extracts of human and chicken red cells revealed that a temperature of 56°C. for 30 minutes usually reduced the titer of inhibition. No detectable inhibition was present after heating at 65°C. for 30 minutes. The inhibitory substance in red blood cell extracts appears therefore to be more susceptible to heat than the substance in serum. It has a greater resemblance in this respect to the substance in liver or muscle extract (Table II). Whether this indicates a fundamental difference between the inhibitor in red cell extracts and that in serum or in certain tissue extracts cannot be determined from the available data. It is well known, however, that the effect of heat on biologically active substances is greatly influenced by a variety of factors, for example the concentration of extraneous proteins, including the kind and quantity of extraneous proteins and other constituents of the menstruum.

*Effect of Removal of Receptor Substance from Red Cells.*—Hirst (1) showed that influenza virus is rapidly adsorbed onto chicken red blood cells and after 4 to 6 hours at 37°C. almost all of the virus is released from the cells. Red cells which had been treated in this manner lost their capacity to agglutinate due to removal of the virus receptor substance from the cells. In the next experiments chicken red blood cells were treated with influenza virus at 37°C. until they no longer agglutinated and then extracts of the cells were tested for capacity to inhibit hemagglutination.

Sterile chicken red blood cells were obtained in citrate and washed three times in sterile saline. 0.5 cc. aliquots of the packed cells were mixed with 19.5 cc. of saline, normal allantoic fluid, PR8, and Lee allantoic fluids, respectively. Although the viruses had been obtained aseptically, penicillin (50 units per cc. final concentration) was added to each mixture as a further precaution. The mixtures were incubated at 37°C. for 16 hours with frequent resuspension of the cells. The red cells were then sedimented by low speed centrifugation and washed twice in sterile saline in the centrifuge. The mixtures were further incubated at 37°C. for another hour. The red cells were then washed twice in saline and suspended in 19.5 cc. of saline. The cells were tested for capacity to agglutinate with influenza and mumps viruses and finally extracted in the Waring blender. The supernatant fluids, after clarification by centrifugation, were tested for residual virus and for the inhibitory substance as described in the preceding experiments.

Chicken red cells treated with saline and normal allantoic fluid retained their capacity to agglutinate with the viruses tested and extracts of these cells caused inhibition of agglutination. Extracts of virus-treated cells, which contained no residual virus and which no longer agglutinated with influenza or mumps viruses, failed to cause detectable inhibition as illustrated in the experiment shown in Table V. The results clearly indicated that removal of the receptor

substance from red cells also removed the inhibitory factor from extracts of the cells.

*Dissociation of Virus from Inhibitory Substance.*—Influenza virus is eluted from chicken red cells after incubation for varying lengths of time at room temperature or, more rapidly, at 37°C. (1). The hemagglutinin of mouse pneumonia virus can be dissociated from the inhibitory component of mouse lung

TABLE VI  
*Dissociation of Influenza Virus from Inhibitory Substance*

Temperature	Units of Lee virus in mixtures	Agglutination of chicken red cells			
		Virus and inhibitor* incubated			
		1 min.	1 hr.	3 hrs.	6 hrs.
22	1	0	0	0	0
	2	0	0	0	0
	4	±	0	0	+
	8	±	0	±	+
	16	+	±	+	+
37	1	0	0	0	0
	2	0	0	0	±
	4	0	0	±	+
	8	±	±	+	+
	16	+	+	+	+

\* Mixtures of chicken red cell extract and virus incubated for various periods as indicated, before addition of red cells (see text for details of experiment).

extracts by heating at 70°C. (7, 8). Experiments were undertaken, therefore to see whether influenza virus could be released from the inhibitory substance in red cell extracts by these methods.

Four cc. of serial dilutions of Lee allantoic fluid in duplicate was mixed with an equal volume of a chicken red cell extract (1:40). One set of the mixtures was placed in a 37°C. water bath, while the other was left at room temperature (23°C.). Samples (0.5 cc.) of each mixture were tested immediately and at 1, 2, 3, and 6 hours, by adding 0.5 cc. of a 0.25 per cent suspension of chicken red cells. Agglutination was recorded as usual after 1½ hours at room temperature.

The results of this experiment are shown in Table VI. Inhibition of agglutination was apparent immediately after mixing the virus with the red cell extract. After incubation for 6 hours, however, hemagglutination was again manifest, particularly in the mixtures containing 4 units of virus. There was no striking difference in the reaction at 23°C. and at 37°C. Repeated tests have confirmed these findings and similar results were obtained with human

red cells. In general, it appeared that the dissociation of virus and the inhibitory substance in the red cell extracts was less striking than the elution of virus from intact red cells (1), and that critical amounts of virus and inhibitor were required for its demonstration.

#### DISCUSSION

The substance (or substances) responsible for inhibition of virus hemagglutination observed in these experiments is apparently widespread in the human and animal body. It can be detected in serum, muscle, various organs, and in the red blood cells of certain species. The experiments with red blood cells (Table IV, V, VI) leave little doubt that the inhibitory substance obtained from these cells is identical with the receptor substance of intact red cells which combines with virus to produce agglutination. It seems likely that the inhibitory effect of tissue extracts is based on the same mechanism, since the virus receptor substance is found in tissue cells as well as in red blood cells. The available data, however, do not exclude the possibility of other substances in tissue extracts producing a similar effect.

The simplest explanation of the non-specific inhibition reaction is that the receptor substance which has been released from cells combines with virus and blocks the union of the virus with the red blood cells. The presence of an inhibitory substance in human tears (6) and also in serum suggests that the receptor substance is released from cells under natural conditions in the body. The findings with the virus inhibitory substance are similar in certain respects to those with the blood group substances A and B. Both are found in many types of mammalian cells in addition to red cells, and also in various body fluids in solution. Furthermore, the blood group substances in solution may combine with isoantibodies to inhibit hemagglutination (12). Evidence that the virus inhibitory substance is not an antibody and that it is distinct from the blood group substances (A, B, and Rh) has been provided in the text.

Further study of the inhibitory substance will be required to assess its significance in the body and in serological tests involving the agglutination inhibition reaction for detecting specific virus antibodies. The failure of the substance to neutralize influenza virus *in vitro* suggests that it is not an important factor in preventing infection. It might be postulated, however, that it plays a rôle in influenza virus infection of individuals with circulating antibodies by preventing union of the virus with antibody. Curnen and Horsfall (8) observed that PVM virus in combination with the tissue factor causing inhibition of hemagglutination failed to fix complement in mixtures with immune serum.

It should be emphasized that a sensitive method for detecting the inhibitory substance was used in the present experiments for purposes of studying the substance. Non-specific inhibition titers of animal sera have been reported to be less than 1:32 when titrated according to the method of Hirst and Pickels

(13), although extracts of ferret lung caused inhibition at a titer of 1:10,000 (3). Salk's method (9) of making serial dilutions of serum in the red cell suspension and then adding virus was found to decrease the non-specific inhibition titer. Apparently, in the latter method, the receptor substance of the red cell and the inhibitory factor in the serum compete for the virus, with less virus bound by the inhibitor than when virus and inhibitor are mixed before the addition of red cells. Francis (14) reported recently that inhibition titers of normal human sera were greatly increased when Type B influenza virus, heated to 56°C., was used instead of unheated virus. It is possible that the factor in serum causing this reaction is the same as the inhibitory substance encountered in the experiments reported here.

At the present time there is no simple method available for differentiating non-specific inhibition from that produced by specific antibody in human sera. The method used to dissociate the hemagglutinin of mouse pneumonia virus from the inhibitory substance in lung extracts, namely heating to 70°C. (7, 8) is not applicable to influenza virus, because the latter is more susceptible to heat than the substance causing inhibition. The dissociation of influenza virus and inhibitor by incubation at 22°C. or 37°C. (Table VI) would not appear to be a practical method for recognizing the non-specific factor, since the reaction proceeds slowly and its demonstration is dependent on critical amounts of virus and inhibitor in the mixtures. A rise in the serum inhibition titer, however, is undoubtedly due to specific antibody, for the fact has been abundantly demonstrated by neutralization and complement fixation tests with influenza virus.

#### SUMMARY

A study of the component in serum and tissue extracts responsible for non-specific inhibition of hemagglutination with mumps virus and the PR8 and Lee strains of influenza virus has yielded the following results:

1. The inhibitory factor was found in high titer in human serum and in saline extracts of various organs procured at autopsy (lung, liver, kidney, spleen). The inhibition titers of extracts of these organs were usually higher than the serum titers, whereas the titers of muscle extracts were invariably lower.

2. Similar results were obtained with serum and tissue extracts from normal rabbits and guinea pigs.

3. The serum inhibition titers were not affected by heating to 75°C. for 30 minutes, whereas the titers of the tissue extracts were usually reduced by heating at 65°C. or 75°C. for 30 minutes.

4. Saline extracts of human and chicken red blood cells also contained an inhibitory substance in high titer, and these cells showed marked agglutination with influenza and mumps viruses. Rabbit red cells, on the other hand,

underwent little or no agglutination with these viruses and extracts of these cells failed to cause inhibition. Sheep red cells varied in their capacity to agglutinate and also in their yield of the inhibitory substance.

5. When the virus receptor substance was removed from chicken red cells by adsorption and elution with influenza virus, extracts of the cells no longer yielded the inhibitory factor.

6. The inhibitory substance did not neutralize influenza virus in mice and it failed to fix complement when mixed with influenza or mumps viruses.

7. Evidence was obtained that some virus was released from the inhibitory substance after incubation for 6 hours at 22°C. or 37°C.

The implications of these findings are discussed.

The technical assistance of Mrs. Jane Brady, Miss Sarah Moorhead, and Miss Frances Sterne is gratefully acknowledged.

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## ANTIPROTEINS IN HORSE SERA

### II. ANTIBODIES TO PNEUMOCOCCUS NUCLEOPROTEIN AND THEIR REACTION WITH ANTIGEN\*†

By MICHAEL HEIDELBERGER, Ph.D.

(From the Department of Medicine, College of Physicians and Surgeons,  
Columbia University, and the Presbyterian Hospital, New York)

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The isolation of bacterial toxins in a state of high purity and their identification as proteins (1) have made it evident that the long known and much studied antitoxins which appear in the sera of toxin-injected horses represent the classical form of antiprotein elaborated by this animal. The *in vitro* reactivity of antitoxins, as typified by the Ramon flocculation titration, is characterized by a narrow zone of specific precipitation with toxins. The region of insoluble compounds corresponds to the equivalence zone (2) of the precipitin reaction. This precipitin type of interaction occurs between a multitude of antigens and homologous antibodies formed in the rabbit, as well as between the specific polysaccharides of pneumococci and the corresponding anticarbohydrate in both rabbit and horse antisera to type-specific pneumococci. The reaction does not show inhibition in the zone of antibody excess.

In view of these differences it became of interest to study the reaction between a typical, non-toxic protein and the antibodies engendered by it in the horse, and an antiserum to crystalline egg albumin (Ea) was prepared (3). This was found to give the reaction pattern of an antitoxin with Ea (3, 4). Subsequently, a similar course was noted for the interaction of hemocyanin and certain samples of antihemocyanin produced in the horse (5). Since, however, the anticarbohydrate in antipneumococcus horse sera gives typical precipitin reactions with the specific polysaccharides of pneumococcus (6), it was thought desirable to characterize, if possible, the reaction of pneumococcus nucleoprotein, one of the group-specific antigens of pneumococci (7), with antinucleoprotein formed in the horse. Many potent antipneumococcus horse sera were tested, but in all of these the immune substances consisted mainly of type-specific anticarbohydrate and smaller quantities of antibodies to "C-substance" (8, 9). Finally, measurable amounts of antiprotein were found in a serum, No. 16,<sup>1</sup>

\* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital.

† The first paper of this series, although not numbered as such at the time, is that given in reference 4.

<sup>1</sup> Kindly made available in 1939 by Dr. Alvan L. Barach, of this Department.

which had been obtained some years previously by intravenous injection of a horse with the pleural exudate resulting from the intratracheal injection of horses with virulent Type I pneumococci (10). The study of this serum is described below.

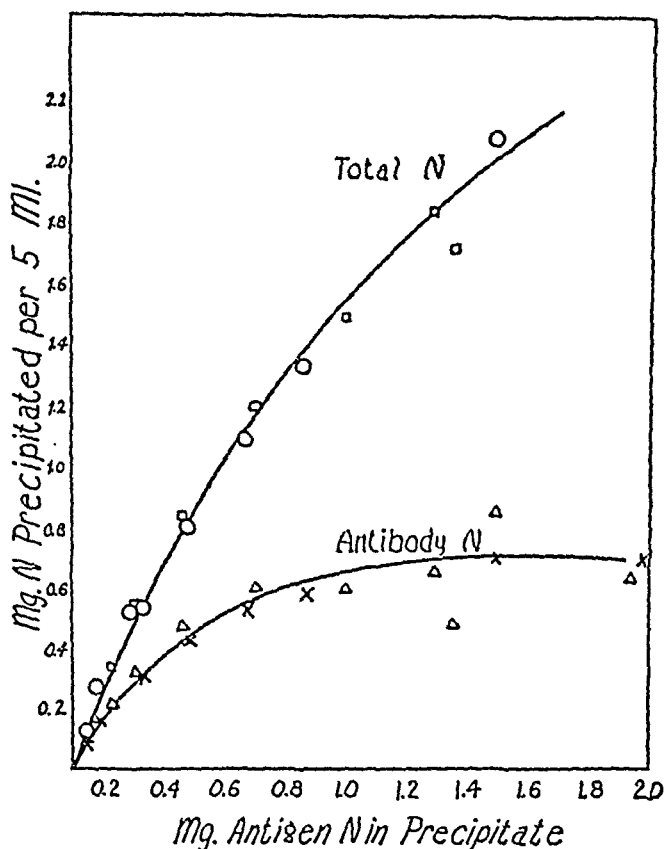


FIG. 1. Squares, circles represent total N in specific precipitate with protein from pneumococcus I R (Dawson S), II R (S), respectively.

Triangles, crosses refer to antibody N precipitated by pneumococcus I, II protein, respectively.

#### EXPERIMENTAL

##### *Materials and Methods*

*Pneumococcus Nucleoprotein*<sup>2</sup>.—R (Dawson S) strains derived from Type I and Type II pneumococci were separately grown for 15 to 20 hours in meat-infusion-phosphate-glucose broth, centrifuged off, and, with or without a preliminary washing with saline, were extracted in the cold for 10 to 12 hours with excess 0.01 N sodium hydroxide solution. After centrifugation the clear supernatants were acidified with acetic acid to maximum flocculation. The precipitates were centrifuged off, redissolved with water and N sodium hydroxide solution until just pink to phenolphthalein, centrifuged, and reprecipitated. After several repetitions of

<sup>2</sup> These preparations were made by Dr. E. A. Kabat in 1933-34.

this process each solution was filtered through a Berkefeld V candle and reprecipitated by means of acetic acid. The product was washed with water and redistilled acetone and dried *in vacuo* at room temperature. Solutions were made by suspension of weighed samples in a little water, addition of a slight excess of N sodium hydroxide, neutralization after maximal solution had been attained, and centrifugation from insoluble material. The resulting solutions were diluted with 0.9 per cent saline and analyzed for nitrogen by the micro-Kjeldahl method. It was assumed, for purposes of calculation, that all of the nitrogen was contained in the nucleoprotein and was available for the precipitin reaction.

TABLE I

*Precipitation of Antipneumococcus Type I Horse Serum 16 with Nucleoprotein from Type I and Type II Pneumococci*

0°C., 48 to 96 hours; calculated to 5.0 ml. serum

Amount Type I pneumo- coccus nucleoprotein nitrogen added	Nucleopro- tein N pre- cipitated*	Total N precipitated	Antibody N precipitated	Antibody N antigen N in pre- cipitate*	Tests on supernatants
mg.	mg.	mg.	mg.		
0.123	Total	0.346	0.223	1.8	Excess A, no NP
0.205	"	0.542	0.337	1.6	" " " "
0.360	"	0.842	0.482	1.3	" " " "
0.600†	"	1.214	0.614	1.0	" " " "
0.900‡	"	1.495	0.595	0.66	Trace A, " "
1.34	1.10¶	1.72	0.62	0.56	Excess NP
3.60**	2.33¶	2.94	0.61	0.26	" "
Amount Type II pro- tein N added					
0.190	Total	0.528	0.338	1.8	Excess A, no NP
0.760	"	1.34	0.58	0.76	No A or NP

NP = nucleoprotein.

\* On the assumption that all of the N added is active as precipitinogen.

† 3.0 ml. serum and equivalent quantity of antigen actually used.

‡ 2.0 ml. serum and equivalent quantity of antigen actually used.

|| 1.5 ml. serum and equivalent quantity of antigen actually used.

¶ After deduction of the quantity of antigen N found in supernatant by analysis of aliquot portions with 5.0 ml. each of fresh serum.

\*\* 1.0 ml. serum and equivalent amount of antigen actually used.

*Antibody Determinations.*—These were carried out quantitatively (6, 11) by addition of accurately measured amounts of nucleoprotein in duplicate to known volumes of the antiserum at 0°C. After the contents were mixed, the tubes containing the analyses were allowed to stand in the cold for 48 to 96 hours, with occasional mixing, and were centrifuged in the cold<sup>3</sup> and washed twice with cold saline. Nitrogen was estimated by a modification of the micro-Kjeldahl method. When antibody was present in excess, the amount of nucleoprotein nitrogen added was deducted from the total N precipitated and the difference was arbitrarily considered antibody N. In the region of excess antigen separate analyses were made of the amount of antigen in the supernatants by addition of these or aliquot portions to fresh por-

<sup>3</sup> In a refrigerated centrifuge manufactured by the International Equipment Co., Boston, Massachusetts.

tions of antiserum. From the quantity of total nitrogen precipitated, the amount of excess antigen could be read from the curve (Fig. 1) in the region of excess antibody and deducted from the total added. The two values in column 2 of Table I were obtained in this way. In the table are given selected data illustrating the course of the reaction with Type I and Type II nucleoprotein, and these and other data are plotted in the curve in the text-figure.

In addition to antiprotein, horse serum 16 contained appreciable antibody to pneumococcus C-substance. This was removed by addition of a slight excess of C-substance derived from Type II pneumococci before any of the analyses were made. The serum also contained 0.8 mg. of antibody nitrogen per ml. to Type I specific polysaccharide, considerably more than the antiprotein content. Prior removal of the type-specific anticarbohydrate led to fluctuations in the quantity of antiprotein precipitated which could not be studied owing to lack of antiserum, but the reaction still retained the characteristics of the precipitin type.

### DISCUSSION

In the production of most antipneumococcus horse sera, intact Gram-positive type-specific pneumococci are injected intravenously, and relatively little antiprotein is produced. However, an exudate was used (10) in the preparation of the serum employed in this study. This presumably contained many autolyzed pneumococci and therefore free nucleoprotein, as well as intact cells, and the appreciable content of antiprotein in the serum is possibly due to this circumstance.

It is apparent from the data in the table and figure that the reaction between pneumococcus nucleoprotein and the antiprotein in this serum was of the precipitin type. It is also to be noted that the antigen had been injected intravenously. The relation of the route of injection to the antitoxin or precipitin type of antibody response in horses will be discussed more fully in the two following papers. However, antitoxic horse sera are usually produced by subcutaneous or intramuscular injections of toxin, and it was similarly found unsatisfactory to employ the intravenous route (3) in the preparation of the anti-egg albumin horse serum in which the flocculation reaction was of the toxin-antitoxin type. It was also determined in a portion dialyzed by Dr. H. P. Treffers against 0.02 M phosphate buffer at pH 6.8 that the antiprotein in antipneumococcus horse serum 16 was mainly in the water-insoluble fraction. This contrasts with the water-soluble character of the antitoxin type of antibody but resembles the anticarbohydrates in horse sera. These also give the precipitin type of reaction.

Little significance is attached to the actual magnitude of the antibody N: antigen N ratios given in Table I, since these were derived by assuming that all of the nucleoprotein nitrogen added was antigenic. This is by no means certain, nor is it likely, from what is known of other bacterial proteins, that the material used was a single substance. A rough attempt at fractionation carried out in this laboratory by Miss Graciela Leyton-Ramirez of Santiago, Chile, showed that the fraction of Type I protein precipitated at one-fifth saturation with sodium sulfate was a slightly more active precipitant of the serum than the

portion separable on acidification of the sodium sulfate supernatant, but the difference was small. Also, preparations of Type I and Type II nucleoprotein behaved remarkably alike toward the antiserum.

#### SUMMARY

The antiprotein in an antipneumococcus horse serum resulting from intravenous injections of infected pleural exudate showed a precipitin type of reaction with pneumococcus nucleoprotein rather than the antitoxin type of response.

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# ANTIPROTEINS IN HORSE SERA

## III. ANTIBODIES TO RABBIT SERUM ALBUMIN AND THEIR REACTION WITH ANTIGEN\*†

By HENRY P. TREFFERS, PH.D., MICHAEL HEIDELBERGER, PH.D., AND  
JULES FREUND, M.D.

*(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital; and the Bureau of Laboratories of the Department of Health, New York)*

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As far as is known, the injection of any antigen into the rabbit by any route may give rise to antibodies which are of the so called precipitin type. Addition of a small amount of antigen to the antiserum results in a precipitate which increases in amount as more antigen is added until the maximum is reached with a slight excess of antigen (2). No "prezone" or inhibition of precipitation in the region of antibody excess is observed. While antibodies to specific carbohydrates may be elicited in the horse or rabbit by intravenous injections of type-specific pneumococci, it is common experience that tetanus or diphtheria antitoxins are produced in the horse in sufficient amounts only in response to subcutaneous injection. These antitoxins flocculate with the antigen, but the precipitation differs from the precipitin type in that it is confined to a relatively narrow zone, inhibition being observed with excess of either antibody or antigen (cf. 3, 4). Antibodies with similar properties have been produced in the horse by the subcutaneous injection of a number of protein antigens: egg albumin (5, 6), hemocyanin (7), and hsiquasin, a crystalline globulin of watermelon seed (8). The few recorded experiments on the intravenous injection of protein antigens into the horse have not been very successful (see (5) for literature).

It was, however, shown in the preceding paper (9) that pneumococcus anti-nucleoprotein in the horse, elaborated after intravenous injections, reacted according to the precipitin type. One or more of at least four factors might be responsible for the qualitative type of antibody produced: the animal species injected, the route of injection, the duration of the immunization, and the nature of the antigen (whether carbohydrate or one or another type of protein). The work about to be described traces the influence of these factors.

Rabbit serum proteins were selected for study as antigens in the horse partly on account of their ready preparation in the relatively large amounts required and partly because information had already been obtained regarding the reverse

\* The work reported in this communication was carried out in part under the Harkness Research Fund of the Presbyterian Hospital, New York.

† A preliminary abstract of a portion of this paper appeared in reference 1.

system, horse serum proteins in the rabbit (10). Rabbit serum was fractionated into albumin and globulin portions, and although neither was homogeneous, it was felt that any resulting multiple antibody response would not be an undue complication since more highly purified test antigens were to be used for the analytical studies of the sera. In some instances, at the termination of a course of injections, it became desirable to change antigens in order to follow the response of an individual animal to the administration of different antigens by the same route. The present report deals with the results obtained with rabbit serum albumin as antigen, while the following paper records the findings with rabbit serum globulin.

## EXPERIMENTAL

### *Methods and Materials*

*Suspensions for Injection.*—Pooled normal rabbit sera were diluted with 2 volumes of water and 1.22 volumes of saturated ammonium sulfate solution were added drop by drop, with stirring to 55 per cent saturation. The precipitate was allowed to stand overnight, was washed once with 55 per cent saturated ammonium sulfate solution, and was reprecipitated in the same way, taken up in water, and dialyzed in the cold until sulfate-free. This solution contained the globulin antigen used.

The supernatants from the two precipitations were mixed and the rabbit albumin was thrown down by addition of acetic acid to maximum turbidity. The precipitate was centrifuged, redissolved in water, and dialyzed against saline in the cold. This solution contained the albumin antigen used.

For injection the albumin or globulin stock solutions, at a concentration of 3.3 mg. of protein per ml., were precipitated by addition of 1 ml. of 1 per cent alum solution per 100 mg. of protein (11), followed by dilute sodium hydroxide to maximum turbidity. One per cent by volume of 1 per cent merthiolate<sup>1</sup> was added as a preservative. For the immunization of horses 1126 and 1127 equal volumes of the albumin and globulin solutions were mixed before precipitation with alum (mixed antigens).

*Injection Procedure.*—The four horses used for these experiments were selected from the regular stock of the Research and Antitoxin Laboratory of the New York City Department of Health, Otisville, New York. All injections and observations on the animals were carried out by members of that Division. The intravenous injections appeared to be better tolerated than the subcutaneous ones, although for all but one animal the dose of protein had to be reduced for a short period during which the horses experienced some rise in temperature. Injection schedules with albumin or albumin-globulin mixtures are given in Table I.

*Test Antigen.*—Rabbit serum albumin was prepared by electrophoretic separation,<sup>2</sup> in a large Tiselius cell, of 90 ml. of rabbit serum which had been dialyzed in the cold for 3 days against buffer at pH 7.7. The electrophoretic homogeneity of the albumin was checked by a separate analytical run.

*Electrophoretic Patterns.*—Electrophoretic patterns were determined on the sera both before and after immunization. The patterns of the postimmunization sera which contained antibody resembled those of typical antitoxic horse sera (12, 13).

<sup>1</sup> Manufactured by Eli Lilly and Co., Indianapolis, Indiana.

<sup>2</sup> In the Electrophoretic Laboratory of the College of Physicians and Surgeons, Columbia University, under the direction of Dr. Dan H. Moore.



*Examination of the Sera for Anti-Albumin*

*Horse 999.*—Precipitin tests, with 0.0002 to 0.04 mg. N of the rabbit albumin test antigen per ml. of serum, on bleedings taken from horse 999 ten days after the last intravenous injection and after a rest period of over 3 months gave no positive result. After the period of rest from the intravenous injections (Table I) horse 999 was given three preliminary intracutaneous injections (< 5 mg. of protein in all) and then eight subcutaneous doses of rabbit serum albumin totalling 1.15 gm. A test bleeding 9 days after the last injection showed a zone of flocculation with albumin. Injections were continued over a period of 5 months and a bleeding was taken 10 days after the last dose.

TABLE I

*Injection Schedule of Horses Receiving Alum-Precipitated Rabbit Serum Albumin or Albumin-Globulin Suspensions*

Horse No.	Antigen	Route of injection	From	To	No. of injections	Average amount per injection
999	Albumin	Intravenous	May 9, 1940	July 2, 1940	13	200
		Subcutaneous	Dec. 4, 1940*	June 24, 1941	41	200†
1046	Albumin	Intravenous	Oct. 28, 1941	May 13, 1942	43	200‡
1126	Albumin + globulin	Intravenous	Oct. 28, 1941	June 12, 1942	48	100†‡
1127	Albumin + globulin	Subcutaneous	Oct. 28, 1941	June 12, 1942	48	100†‡

\* The first three injections (0.1 to 3.6 mg.) were given intracutaneously.

† Dosage reduced for four injections because of febrile reaction.

‡ Dosage increased continuously by 10 mg. per injection from 5 mg. to 385 mg.

|| Albumin portion only; an equal amount of globulin was also present.

Quantitative estimations of total N and antibody N precipitated (Table II) were carried out as in previous studies (14). In a typical instance 5.0 ml. amounts of the serum were measured out at 0°C., and appropriate amounts of electrophoretically separated rabbit serum albumin were added in accordance with preliminary tests on smaller volumes. The volume was adjusted to 9.5 ml. with saline and the contents of the tubes were carefully mixed and allowed to stand either 3 or 7 days in the ice box, with mixing at intervals. The tubes were centrifuged in the cold,<sup>2</sup> the precipitates were washed three times with 4 ml. portions of saline at 0°, and were then analyzed for nitrogen by a modification of the micro-Kjeldahl technique. In one instance the supernatants from the tubes which had stood 7 days were allowed to remain in the ice box for another week, centrifuged, and the precipitates washed. The nitrogen found was added to the corresponding values for the 7 day experiment. Antibody N was estimated by subtracting the added antigen N from the total nitrogen found, under the assumption that all added antigen N was precipitated (*cf.* 2).

<sup>2</sup> In a refrigerated centrifuge supplied by the International Equipment Co., Boston, Massachusetts.

In the series which was allowed to stand for 3 days, the largest amounts of total N precipitated from 5 ml. of the July (later) and January (earlier) bleedings were 1.06 and 0.430 mg., respectively; ratio, 2.47. In the experiment with the January bleeding, by multiplication of each value of the antigen N added and the total N precipitated by 2.47 the behavior of this serum may be compared directly with that of the later, stronger bleeding (Fig. 1).

TABLE II

*Precipitation of Horse Antibody to Rabbit Serum Albumin by Electrophoretically Separated Rabbit Serum Albumin, per 5.0 Ml. Serum, Horse 999, 0°C.*

Antigen N added	Total N* precipitated after			Antibody N precipitated‡
	3 days	7 days	2 wks.	
mg.	mg.	mg.	mg.	mg.
Bleeding Jan. 23, 1941				
0.040	0			
0.050	0.116			0.066
0.060	0.303			0.243
0.080	0.394			0.314
0.100	0.430			0.330
0.120	0.422			0.302
0.150	0.156			
0.200	0			
Bleeding July 2, 1941				
0.096	0.256	0.524	0.586	0.490§
0.144	0.954	0.938	0.940	0.796
0.169	0.990	0.913	0.923	0.754
0.192	1.02	0.984	0.984	0.792
0.240	0.986	1.05	1.06	0.82
0.288	0.994	1.05	1.06	0.77
0.336	0.486	0.552	0.586	
0.384	0.018			

\* The 3- and 7-day columns represent independent series run in duplicate; column 4 contains data obtained by adding to the values in the preceding column one-half of the small amount of nitrogen which separated from the combined supernatants of each pair of tubes in the 7-day series after an additional week.

† All added antigen N assumed to be in the precipitate.

§ Values in column 4 minus corresponding values in column 1.

|| 4.25 ml. serum and 0.144 mg. albumin N actually used.

In order to study the effect of volume on the precipitability of the antibody a concentrate was prepared from the July bleeding. The fraction precipitated from 500 ml. of serum by one-third saturation with ammonium sulfate was removed, washed, and discarded since it contained little antibody. The supernatants and washings were brought to  $\frac{1}{3}$  saturation with ammonium sulfate. The resulting precipitate was taken up in water and dialyzed against water in the cold until free from sulfate. After removal of the water-insoluble portion, the supernatant was concentrated by dialysis under negative pressure to a final volume of 115 ml.

Portions of this globulin concentrate, 999 B, were set up at 0° against electrophoretically separated rabbit serum albumin test antigen. In one series the total volume was kept at

3 ml., in the other at 10 ml. After 6 days in the ice box both series were centrifuged, given three washings with 3 ml. of cold saline, and analyzed as before. The data are given in Table III:

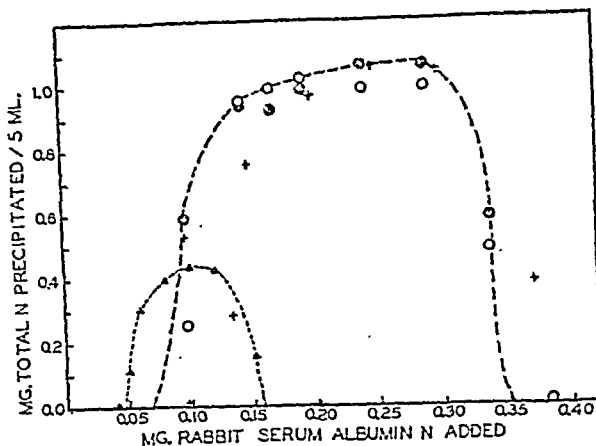


FIG. 1. Precipitation of horse antibody to rabbit serum albumin by electrophoretically separated rabbit serum albumin. Triangles = Jan. 23, 1941, bleeding; crosses = experimental values  $\times 2.47$  for comparison with later (July 2, 1941) bleeding. Open circles = July 2, 1941, bleeding, 3 day experiment; shaded circles = same, 14 day experiment.

TABLE III

*Effect of Volume on the Precipitation of Horse Anti-Rabbit Serum Albumin*

Per 1.0 ml. globulin solution, 999B, bleeding of July 2, 1941, 0°, 6 days. Test antigen: electrophoretically separated rabbit serum albumin

Antigen N added	Total N precipitated at volume of 3 ml.	Total N precipitated at volume of 10 ml.	Antigen N added	Total N precipitated at volume of 3 ml.	Total N precipitated at volume of 10 ml.
mg.	mg.	mg.	mg.	mg.	mg.
0.048	0.020	0.012	0.160	0.694*	0.688
0.060	0.140	0.134	0.171	0.698	0.648
0.072	0.502	0.410	0.192	0.636	0.526
0.096	0.612	0.566	0.216		0.334
0.128	0.662	0.652	0.233	0.158	0.052
0.144	0.678		0.274	0.054	0.01

\* Ratio antibody N: antigen N at maximum precipitation: 3.3.

*Sera from Horses 1126 and 1127.*—Two other horses were injected (Table I) in order to test the generality of the findings. Horse 1126 received intravenously a mixture of the rabbit albumin and globulin, while No. 1127 received the same antigens subcutaneously. Only the results obtained on testing with rabbit albumin will be noted here, those dealing with globulin test antigens being reserved for the paper following. After twenty-two injections a test bleeding on horse 1126 taken Dec. 16, 1941, showed a zone of weak precipitation with electrophoretically separated rabbit albumin extending over at least an eightfold range of

antigen concentration. A single absorption of this serum was carried out with an egg albumin anti-egg albumin (rabbit) specific precipitate which, containing rabbit  $\gamma$ -globulin in an insoluble form, served as a test antigen for antibody to globulin. The added specific precipitate was visibly agglutinated and the reaction with the rabbit serum albumin was noticeably diminished in the supernatant. It is therefore likely that precipitation in this bleeding was due to a small amount of globulin present in the albumin test antigen. The serum from horse 1127 did not give any reaction at this time. Test bleedings taken Feb. 4, 1942, indicated, however, that the subcutaneously injected horse 1127 was producing anti-albumin of the antitoxin type, like horse 999. This is illustrated by quantitative data given below.

*Effect of Temperature on the Precipitation of Anti-Albumin.*—An antibody concentrate was made from 300 ml. of a bleeding of horse 1127 taken at the conclusion of the immunization schedule. The portion precipitated by  $\frac{1}{2}$  saturation with ammonium sulfate was separated by dialysis against distilled water into a water-insoluble fraction (1127G) and a water-soluble

TABLE IV

*Effect of Temperature on the Reaction of Horse Anti-Rabbit Serum Albumin. Precipitation of 2.0 Ml. of Globulin Solution 1127J by Electrophoretically Separated Rabbit Serum Albumin*

Antigen N added  mg.	Total N precipitated	
	0°C., 7 days  mg.	37°C., 3 hrs.  mg.
0.048	0*	0.002
0.073	0.312	0.166
0.098	0.506	0.490
0.110		0.542
0.146	0.616	0.632
0.171	0.574	0.602
0.219	0.006	0.126

\* One determination only.

portion (1127H). The protein which precipitated between  $\frac{1}{2}$  and  $\frac{1}{3}$  saturation was similarly divided into a water-insoluble part (1127I) and a water-soluble fraction (1127J). Tests indicated that most of the anti-albumin was in fraction J.

2.0 ml. portions of 1127J were analyzed with various amounts of antigen in a total volume of 4 ml. One series of analyses was set up at 0° and allowed to stand in the ice box for 1 week, with mixing at intervals, followed by centrifugation and washing in the cold. A second series was run at 37° and left in the water bath for 3 hours before centrifugation and washing at 37°. The data for both series are given in Table IV.

*Reversibility of Rabbit Serum Albumin Anti-Albumin Combination.*—The reversibility of the antigen-antibody compounds formed on either side of the precipitating region (*cf.* Table IV) was tested by addition, at both 0° and 37°, to duplicate portions of 1127J, of amounts of antigen which would not give visible precipitation. In one case insufficient antigen for precipitation was added, in the other an excess of antigen calculated to inhibit precipitation completely. After 3 to 48 hours, depending upon the temperature, additional antigen or serum was added in amount calculated to bring the system to the point of maximum precipitation (corresponding to the addition of 0.146 mg. of antigen N for each 2.0 ml. of solution J). The tubes were then allowed to stand the same length of time as the controls treated initially with the optimum amount of antigen. The data, summarized in Table V, indicate the reversibility of the system.

*Determination of the Chemical Nature of the Anti-Albumin with an Anti-Antibody Serum.*—In a previous communication (10) a study was reported of quantitative aspects of the observation that antibodies to crystalline egg albumin and diphtheria antitoxin in the horse could be distinguished serologically from typical antibacterial antibodies such as pneumococcus anti-carbohydrate. When these antibodies, in the form of specific precipitates, were used as antigens much as bacterial suspensions in the quantitative agglutination procedure (15) it was found that the first pair removed only 50 to 60 per cent of the antibody in a rabbit antiserum to Type II pneumococcus anticarbohydrate from the horse.

TABLE V  
*Reversibility of Albumin Anti-Albumin Interaction*

Temperature	Volume of antibody solution (1127) used	Rabbit serum albumin N added	Time allowed	Appearance	Second reagent	Further time allowed	Antibody N precipitated
°C.	ml.	mg.	hrs.			hrs.	mg.
0	2.0	0.024	48	Clear	0.122 mg. antigen N	168	0.600*
0	1.0	0.146	48	Clear	1.0 ml. antibody solution	168	0.610
0	2.0	0.146		Precipitate		168	0.616†
37	2.0	0.024	0.5	Clear	0.122 mg. antigen N	3	0.616
37	1.0	0.146	0.5	Clear	1.0 ml. antibody solution	3	0.634
37	2.0	0.146		Precipitate		3	0.632†

\* One determination lost.

† Control determination from Table IV.

Since the antibody to rabbit serum albumin resembled diphtheria antitoxin and anticyrystalline egg albumin in its water-solubility and its flocculation with the homologous antigen it appeared desirable to compare the antigenic properties of the three antibodies. A rabbit antiserum to specific precipitate from the capsular polysaccharide of Type II pneumococcus and Type II antipneumococcus horse serum (10) could not be directly absorbed with the albumin anti-albumin floccules, however, since the unfractionated serum contained rabbit albumin which might interfere with the reaction by a solvent effect on the floccules. An antibody globulin solution was therefore prepared from this rabbit serum by twice repeated precipitation with an equal volume of ammonium sulfate, with thorough washing of the precipitate to free it from as much albumin as possible. After dialysis to remove ammonium sulfate, the antibody globulin solution was analyzed with a floccule suspension prepared from serum 999 (Jan. 23, 1941) by precipitation with rabbit albumin, and for comparison, with a Type I pneumococcus specific precipitate from horse serum, with results given in Table VI.

The anti-albumin floccules removed 0.19 mg. of antibody N, or 45 per cent of the total present. As a check, the supernatant was further absorbed with pneumococcus specific precipitates. In view of the number of absorptions, the total, 0.37 mg. N, is in satisfactory agreement with the direct determination of homologous antibody N, 0.42 mg.

*Intravenous Injection of Albumin.*—It will be noted from Table I that three horses, 999, 1046, and 1126 received rabbit serum albumin intravenously during at least part of the immu-

nization period. Serum taken from horse 999 after a short course (thirteen injections) gave no precipitate over a range of dilutions of the test antigen. Serum from horse 1016 (bleeding May 14, 1942) after an extensive series of intravenous injections, and from horse 1126 which had received smaller intravenous amounts of albumin, together with globulin, showed some flocculation with rabbit albumin, but compared with the serum of horse 1127 which had received a parallel subcutaneous course (Table I) the zones were much broader and the precipitates, even at the maximum, were much smaller. Globulin concentrates of these final bleedings of horses 1016 and 1126 were also prepared, but the portions precipitated at  $\frac{1}{2}$  saturation (1046A and 1126C) and  $\frac{1}{2}$  to  $\frac{1}{2}$  saturation with ammonium sulfate (1046B and 1126D) were not further fractionated as had been done for serum 1127. For antibody tests on the fractions, portions of the flocculating concentrate 1127J were set up with several amounts of albumin corresponding to a point near the maximum of precipitation (Fig. 1), a point on the descending part of the curve, and a point just beyond it. When the precipitation was carried out in the

TABLE VI

*Removal of Antibody from an Anti-Antibody (Pn II Horse Specific Precipitate) Rabbit Globulin Solution by Specific Precipitates from Horse Sera*

Suspension used	Antibody N precipitated			
	First absorption	Second *	Third *	Total
	mg.	mg.	mg.	mg.
H 999 floccules.....	0.184	0.004	0	0.19
Pn I specific precipitate (on supernatant of last absorption above)	0.132	0.036	0.012	0.18
				0.37
Pn I specific precipitate.....	0.342	0.077	0.005†	0.42

Pn used for pneumococcus.

\* Single determinations on combined supernatants.

† Pn II specific precipitate suspension used for this absorption.

presence of concentrates 1046A and B, and 1126C and D, only 1046A failed to show some deviation of the zone of flocculation as compared with that of the control containing 1127J alone. All of the concentrates had been absorbed twice with rabbit anti-egg albumin specific precipitates to free them of at least part of their anti- $\gamma$ -globulin antibody. The presence of some antibody in the sera of horses 1046 and 1126 was therefore indicated. However, quantitative precipitin tests of 2 ml. portions of solutions 1046B and 1126D with six different amounts of albumin antigen yielded precipitates whose total N ranged from 0.012 to 0.112 mg. Since this was only 0.6 to 0.2 of the N added in the form of antigen, it is evident that the reaction was not due to the rabbit serum albumin itself, but most likely to some impurity, possibly traces of  $\alpha$ - or  $\beta$ -globulin, all or most of the antibody to  $\gamma$ -globulin having been previously absorbed.

#### DISCUSSION

The data show that horses 999 and 1127, which had been injected subcutaneously with rabbit serum albumin, yielded antibody of the flocculating type, with inhibition zones in the regions of antibody and antigen excess. In this respect the anti-rabbit serum albumin resembled other antiproteins elaborated by the horse, except the antinucleoprotein described in the preceding paper (9).

The electrophoretic patterns of the sera of horses 999 and 1127 clearly showed the appearance, after the subcutaneous injections, of a new component migrating between the  $\beta$ - and the  $\gamma$ -globulins. Similar changes have characterized antitoxic sera in horses (12, 13), as well as some antipneumococcus sera (16).

The first course of injections for horse 999 was given intravenously and no precipitation was observed in serum taken after the course. The succeeding subcutaneous course of injections evoked a prompt response, and it is readily seen (Table II) that the newly formed antibody was of the flocculating type. As is evident from the data on the later bleeding reported in Table II and Fig. 1, the amount of nitrogen precipitated in the cold increased appreciably when the tubes were allowed to stand for longer periods than customarily used, especially when the proportions of antigen and antibody deviated notably from those giving maximum precipitation. It is quite possible that the shape of the curve for the earlier bleeding might have been somewhat altered if the precipitations had been allowed to stand longer.

If the assumption is made that all of the antigen N added is precipitated in the equivalence region, antibody N values may be calculated by subtraction from the total N. In the first bleedings (Table II) the antibody N remained constant (0.30 to 0.33 mg.) when the amount of antigen N added was varied by 50 per cent. Similarly, for the later bleeding, the antibody N varied between 0.82 and 0.77 mg. when the amount of antigen was increased from 0.144 to 0.288 mg. N.

This behavior appears to be characteristic of the flocculation reaction, having been observed as well in the diphtheria antitoxin (4), scarlatinal antitoxin (17), and anti-egg albumin systems in horse sera (5, 6). In all of these there is a region in which the total N precipitated increases linearly with added antigen. The amount of antibody precipitated is the same throughout this region, however, since the increase in total N is due to the precipitation of antigen, all of which is precipitated if homogeneous. In this region the reaction resembles the usual precipitin reaction (*i.e.*, a system without an inhibitory prezone) in that the ratio of antibody N to antigen N precipitated decreases as more antigen is added. There is, however, the distinction that in the precipitin type of reaction the antibody N precipitated usually increases slightly as well.

Pappenheimer and coworkers (4, 17) have used the constancy of the amount of antibody precipitated in the floccules to determine the amount of nitrogen corresponding to a flocculating unit of toxin or to determine, by difference, the per cent precipitability of a flocculating antigen. Similar calculations may be made from the present data. It will be noted from Table III (3 ml. series) that 0.096 mg. of albumin N precipitated 0.612 mg. of total N, while 0.171 mg. of antigen N removed 0.698 mg. N. The increase in total N precipitated, 0.086 mg., is the same, within experimental error, as the increase in the amount of added antigen N, 0.075 mg. The corresponding difference in the total N pre-

precipitated in the 10 ml. series is 0.082, again evidence that all of the albumin N added was effectively antigenic. Similarly, in Table II an increase of 0.144 mg. antigen N resulted in an increase of 0.12 mg. total N precipitated.

The data of Table IV, obtained with antibody concentrate 1127J, are somewhat at variance with this, since  $0.146 - 0.098 = 0.048$  mg. antigen N resulted in an increase of 0.11 and 0.14 mg. of total N at  $0^{\circ}$  and  $37^{\circ}$ , respectively. This disproportionate increase in the amount of N precipitated is as yet unexplained.

An early bleeding of horse 999 (January 1, 1941) (Table II) and a later one (July 2, 1941), were compared quantitatively by multiplying the antigen N added and the total N precipitated in the earlier bleeding by 2.47, the ratio found for the total N precipitated (as well as the relative antibody contents) in the two bleedings. It is evident that the points calculated for the earlier bleeding (crosses, Fig. 1) lie quite close to the experimental curve for the later bleeding, at least in the linear region, and that the two maxima occur with the same amount of antigen. Although the heights of the two curves can be made to coincide arbitrarily it does not necessarily follow that the maxima will occur with the same amounts of antigen unless the combining ratios are the same. These may be estimated directly by calculation of the antibody N to antigen N ratios, at for example, the midpoints of the flocculation zones (Table II). A value of 3.3 is obtained for the earlier bleeding compared with 4.1 to 3.4 for the later bleeding, depending upon the mid-point chosen. It is evident, therefore, that the combining properties of the antibody did not change greatly on continued immunization of the animal, as in some other systems (6, 11) even though the absolute quantity of antibody increased 2.5-fold.

Within the time limit selected, 6 days, precipitation at the maximum was not influenced (0.694 to 0.688 mg. total N) by variation of the reaction-volume from 3 to 10 ml. (Table III). This did not apply to points on either side of the maximum, as these usually showed less precipitation at the greater dilution.

In the experiments at  $0^{\circ}$  and  $37^{\circ}$ , carried out with antibody concentrate 1127J, the points of maximum precipitation coincided in both series, but more N was precipitated in the region of antibody excess at  $0^{\circ}$  than at  $37^{\circ}$  (Table IV). The reverse was true in the region of antigen excess. Whether these effects are due to varying rates of attainment of equilibrium at the two temperatures, or to real changes in the combining proportions, it is evident that the amount precipitated at the maximum is the same at either temperature, and that in this respect the system resembles diphtheria toxin-antitoxin (18) and the egg albumin anti-egg albumin reaction (5, 6) in the horse, as well as protein-antiprotein (11) systems in the rabbit. It will be recalled that carbohydrate-anticarbohydrate reactions in horse sera may show quite pronounced temperature effects. (19) and, as will be discussed in the following paper (20), this is true for some antiprotein reactions in this species as well.

The data given in Table V show that the soluble antigen-antibody complexes



formed in the two zones of inhibition (for diphtheria toxin-antitoxin, *cf.* (21)) react reversibly with more of the component not in excess, since addition of this component in proper amount suffices to bring the system to the point of maximum precipitation.

Although quantitative estimations were not made on all globulin fractions of the serum of horse 1127, it was evident from qualitative flocculation tests that most, if not all, of the antibody to the rabbit serum albumin was present in the water-soluble portions, particularly of the fraction precipitating between  $\frac{1}{3}$  and  $\frac{1}{2}$  saturation with ammonium sulfate. This fraction of the serum of horses immunized with diphtheria toxin or toxoid also contains most of the antitoxin. Antibacterial horse sera, on the other hand, contain a much larger proportion of water-insoluble antibodies, and antibody to the type-specific carbohydrate of pneumococcus may be almost quantitatively recovered by dilution with slightly acid water (22).

Since the chemical properties of the antibody to rabbit serum albumin, as well as its behavior toward its homologous antigen (Tables II to IV) suggested that it closely resembled diphtheria antitoxin in the horse, it was of interest to see whether the correlation held for its serological activity toward an anti-antibody serum. The latter, produced by injecting rabbits with a specific precipitate derived from Type II antipneumococcus horse serum, had previously been shown to differentiate antitoxic from antibacterial antibodies produced in the horse (10). As is demonstrated in Table VI, the antibody to rabbit serum albumin, in the form of floccules with its antigen, removed only about one-half of the antibody present, as did diphtheria toxin-antitoxin floccules. This serological criterion, therefore, also emphasizes the similarity between the subcutaneously elicited anti-rabbit serum albumin and the similarly induced diphtheria antitoxin and anti-egg albumin in the horse. In agreement with the practical experience of others in the production of antitoxin, intravenous injection of horses with the same antigen did not result in detectable production of antibody with these characteristics. It is, however, possible that antibody is produced after intravenous injection in a form not detected by these tests, and it is hoped to undertake further examination of the sera with this in mind.

While, with the exception of hemolysin (23), antibody produced in the rabbit appears to be of only one qualitative type, it is clear that even in this species the response to a given antigen may be markedly influenced by the route of injection. The intracutaneous or subcutaneous injection of pneumococci (24) and of streptococci (25) results mainly in the production of species-specific antinucleoprotein. Type-specific antibodies may be produced abundantly, however, on intravenous injection of these microorganisms.

Thus far, in the horse, the production of the zonal flocculating type of antibody appears to have occurred only after subcutaneous injection. This, however, is not the only type of anti-protein response by the subcutaneous route, as will be set forth in detail in the following paper (20).

## SUMMARY

1. Two horses were injected subcutaneously with alum-precipitated rabbit serum albumin.

2. The resulting antibody resembled diphtheria antitoxin and anti-egg albumin in the horse in giving a sharp zone of flocculation with antigen, in being water-soluble, in reactivity toward an anti-antibody rabbit serum, and in its electrophoretic properties.

3. The effect of continued immunization, and of variation in volume and temperature on the reactivity of the antibody are discussed.

4. Intravenous injection of the same antigen into horses did not give rise to detectable amounts of antibody of the same type.

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## ANTIPROTEINS IN HORSE SERA

### IV. ANTIBODIES TO RABBIT SERUM GLOBULIN AND THEIR INTERACTION WITH ANTIGEN\*†

By HENRY P. TREFFERS, PH.D., MICHAEL HEIDELBERGER, PH.D.,  
AND JULES FREUND, M.D.

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital; and the Bureau of Laboratories of the Department of Health, New York)

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In the preceding paper (2) it was shown that the subcutaneous injection of horses with rabbit serum albumin was followed by the production of antibody. This antibody resembled the diphtheria antitoxin and anti-egg albumin formed in horses in its solubility in water, its limited zone of flocculation with antigen, and its serological behavior toward an anti-antibody serum. It was also shown that precipitable antibody was not produced following intravenous injections of the same rabbit serum albumin.

Nevertheless, it had been found that intravenous injection of pneumococcal lung autolysates into horses elicited the formation of antibody to the pneumococcal protein (3), and that this antibody combined with antigen in a typical precipitin reaction devoid of the prezone characteristic of the reactions of the antitoxins and anti-albumins. It is now shown that intravenous injection of horses with another kind of protein antigen—in this instance rabbit serum globulin—results in the production of antibody giving a typical precipitin reaction. In addition, intracutaneous or subcutaneous injection of horses with rabbit serum globulins gives rise to antibody of the so called "univalent" (4), soluble, low grade, non-flocculating type.

#### EXPERIMENTAL

*Injection of Horses.*—The preparation of the alum-precipitated rabbit serum globulin and the mixed albumin-globulin suspensions used as antigens was described in reference 2. The four horses used were from the regular stock of the New York City Department of Health Research and Antitoxin Laboratory, Otisville, New York. The schedule of injections is given in Table I.

*Electrophoretic Patterns.*—Electrophoretic runs<sup>1</sup> were made with the sera of the horses injected with rabbit globulin. In the case of horse 1046 the areas of the  $\gamma$ -components were

\* The work reported in this communication was carried out in part under the Harkness Research Fund of the Presbyterian Hospital, New York.

† A preliminary abstract of a portion of this paper appeared in reference 1.

<sup>1</sup> In the Electrophoretic Laboratory of the College of Physicians and Surgeons, under the direction of Dr. Dan H. Moore.

essentially the same before and after injection, 22.8 per cent and 24.8 per cent of the total, respectively. After intravenous injection with mixed albumin-globulin, the serum of horse 1126 gave a pattern in which the  $\gamma$ -peak was prominent. Neither postimmunization pattern showed the pronounced peak between the  $\beta$ - and  $\gamma$ -globulins which characterized the electrophoretic diagrams of the sera of horses 999 and 1127 after subcutaneous injection with albumin or albumin-globulin mixtures (2).

TABLE I

*Injection Schedule of Horses Receiving Alum-Precipitated Rabbit Serum Globulin or Albumin-Globulin Suspensions*

Horse No.	Details of injection					
	Antigen	Route	From	To	No. of injections	Average amount
1046	Globulin	Intravenous	Dec. 31, 1939	July 5, 1940	64	45*
		Intracutaneous	Dec. 4, 1940	Mar. 7, 1941	32	25
		Subcutaneous	Mar. 10, 1941	July 2, 1941	38	200†
		For later immunization see Table I, reference (2).				
999	Globulin	Subcutaneous	Oct. 28, 1941	June 9, 1942	51	240
1126§	Globulin + albumin‡	Intravenous	Oct. 28, 1941	June 12, 1942	48	100
1127§	Globulin + albumin	Subcutaneous	Oct. 28, 1941	June 12, 1942	48	100

\* The first seventeen injections were of 100 mg. globulin each, the remainder 25 mg., with few exceptions.

† The dosage was gradually increased from 25 to 200 mg. during the first nine injections and then held at that level.

§ From Table I, reference 2.

|| Dosage reduced for four injections because of febrile reactions. Dosage of globulin only; an equal amount of albumin was also present.

### *Examination of Sera for Antibody*

*Intravenous Series.*—Three and one-half months after the start of the injections, serum from horse 1046 (bleeding Mar. 18, 1940) showed precipitating antibody. A concentrate of the total globulin was prepared from 1950 ml. of this serum by precipitation with ammonium sulfate and dialysis in the cold against 0.9 per cent NaCl. The final volume was 500 ml.

Antibody in this concentrate was determined by the quantitative precipitin method (5, 2), with  $\gamma$ -globulin electrophoretically separated from normal rabbit serum as test antigen. The results are recorded in Table II. Antibodies to rabbit serum components other than  $\gamma$ -globulin were undoubtedly present, since the total globulins, containing some albumin as well, were used as immunizing antigen. Tests with  $\alpha$ - and  $\beta$ -globulins were made, but could not be interpreted since it was difficult to secure enough of these components to ensure at least their electrophoretic homogeneity. The rabbit  $\gamma$ -globulin, on the other hand, could be obtained in larger quantities. Its essential homogeneity in the Tiselius apparatus was demonstrated by a second mobility determination. Moreover, the results obtained could be

compared with those secured with antigens consisting of specific precipitates containing  $\gamma$ -globulin antibody.

Qualitative precipitin tests with the final bleedings of horse 1126 (Table I) indicated that these sera were similar in their behavior to the corresponding bleedings of horse 1046.

*Influence of Temperature on the Reactivity of Antibody.*—Two series of precipitin determinations were made on whole serum of horse 1046 at the conclusion of the intravenous schedule. One was set up at 0°C. and the tubes were allowed to stand 3 days in the ice box, with subsequent washing in the cold.<sup>2</sup> The other series was set up at 37°C. and the tubes were incubated for 2½ hours and centrifuged and washed at the same temperature. The data are given in Table III and plotted in Fig. 1.

*Determination of Antibody by Absorption with Specific Precipitates.*—Since anti-egg albumin in the rabbit is known to be a  $\gamma$ -globulin (6) and specific precipitates containing such antibody,

TABLE II

*Precipitation of Horse Antibodies to Rabbit Globulin by Electrophoretic Rabbit  $\gamma$  Globulin*  
Per 8.0 ml. concentrate of serum 1046, bleeding Mar. 18, 1940, 0°C., 48 hours

Antigen N added	Antigen N precipitated	Total N precipitated	Antibody N precipitated (by difference)	Antibody N / Antigen N	Tests on supernatants
mg.	mg.	mg.	mg.		
0.019 <sub>2</sub>	Total*	0.144	0.125	6.5	Excess HA†
0.038 <sub>1</sub>	"	0.242	0.204	5.3	" "
0.057 <sub>6</sub>	"	0.300	0.242	4.2	" "
0.077	"	0.350	0.273	3.5	No HA or $\gamma$ G
0.096	0.088§	0.382	0.294	3.3	Excess $\gamma$ G

Mg. antibody N precipitated = 9.3 ( $\gamma$ G N) — 20.6 ( $\gamma$ G N)<sup>1/2</sup>

Calculated to 1.0 mg. precipitable antibody N:

Mg. antibody N precipitated = 9.3 ( $\gamma$ G N) — 10.9 ( $\gamma$ G N)<sup>1/2</sup>

\* All N of the gamma globulin solution was assumed to be antigenically active.

† HA = horse antibody;  $\gamma$ G N = rabbit electrophoretic gamma globulin N.

§ From analyses on supernatant.

if properly washed, can be obtained free from other serum proteins (7), they provide a convenient source of  $\gamma$ -globulin, suitable for use as antigen (8, 9). The analytical procedure is similar to that of the quantitative agglutinin method (10): an accurately measured amount of a suspension of the washed specific precipitate, of known N content, is added to duplicate portions of serum. If the serum contains much antibody visible agglutination often occurs after the contents of the tubes are mixed. The tubes are centrifuged, washed 2 to 3 times with 0.9 per cent NaCl, and the precipitates analyzed for N. The excess of N found over that added is taken as antibody N. Absorption with fresh portions of precipitate is continued until no more antibody N is added. Since the antigen is virtually insoluble, inhibition reactions due to excess antigen are avoided.

Bleedings from horses injected by the various routes were analyzed by this method. A carefully washed, anti-egg albumin egg albumin specific precipitate with a high antibody to antigen ratio was used as antigen, with the results given in Table IV.

<sup>2</sup> In a refrigerated centrifuge supplied by the International Equipment Co., Boston, Massachusetts.

TABLE III

*Effect of Temperature on the Precipitation of Horse Antibodies to Rabbit Globulin by Electrophoretic Rabbit  $\gamma$  Globulin*

Per 5.0 ml. horse serum II 1046, bleeding July 25, 1940, 0° and 37°C.

Antigen N added	Total N precipitated	Antibody N precipitated (by difference)	$\frac{\text{Antibody N}}{\text{Antigen N}}$	Tests on supernatants
mg.	mg.	mg.		
0° C., 3 days				
0.006,*	0.074	0.067	10.3	Excess A
0.013*	0.123	0.110	8.5	" "
0.022†	0.172	0.150	6.8	" "
0.033†	0.190	0.157	4.8	" "
0.039	0.206	0.167	4.3	" "
0.043†	0.217	0.174	4.0	Slight excess A, tr. $\gamma$ G?

Mg. antibody N precipitated = 14.4 ( $\gamma$ G N) - 52.0 ( $\gamma$ G N)<sup>3/2</sup>. Calculated to 1.0 mg. antibody N: Mg. antibody N precipitated = 14.4 ( $\gamma$ G N) - 21.0 ( $\gamma$ G N)<sup>3/2</sup>

37°C., 2½ hours				
0.013	0.084	0.071	5.5	Excess A
0.022†	0.120	0.098	4.5	" "
0.033†	0.140	0.107	3.2	" "
0.043†	0.158	0.115	2.7	" "
0.052†	0.175	0.123	2.4	No A, tr. $\gamma$ G

Mg. antibody N precipitated = 8.6 ( $\gamma$ G N) - 28.5 ( $\gamma$ G N)<sup>3/2</sup>.

$\gamma$ G N = electrophoretic rabbit  $\gamma$ -globulin N.

\* 10 ml. serum actually used for analysis.

† 6 ml. " " " " "

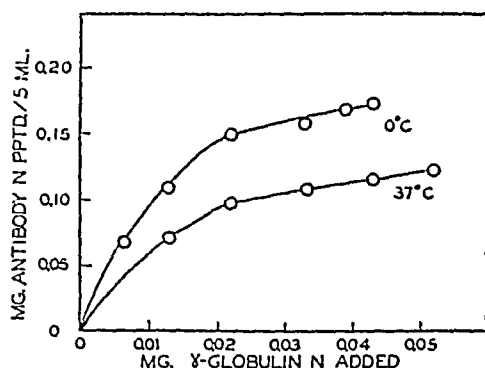


Fig. 1. Effect of temperature on the precipitation of horse antibodies to rabbit serum globulin by electrophoretically separated rabbit serum  $\gamma$ -globulin.

[Sera Obtained after Intracutaneous and Subcutaneous Injections of Globulins.—Horse 1046 was rested for 5 months after the intravenous injections and then received a course of intra-

cutaneous injections of the same antigens (Table I). Although a bleeding taken Mar. 10, 1941, contained 0.11 mg. of antibody N per 5 ml. when analyzed with particulate  $\gamma$ -globulin antigen as above, it failed to precipitate with any of a number of dilutions of rabbit  $\gamma$ -globulin solution. This indicated that the antibody was univalent (4) or low grade.

Horse 1046 was next given subcutaneous injections of the rabbit serum globulins. Bleedings were taken July 2, 1941, and Oct. 28, 1941. The latter bleeding was negative, but the July 2, 1941, bleeding gave slight precipitation with relatively large amounts of  $\gamma$ -globulin (0.025 to 0.125 mg. N). Since the quantity of antigen used for maximum precipitation was

TABLE IV

*Antibody Content of Anti-Rabbit Globulin Horse Sera as Determined by Quantitative Absorption with Anti-Egg Albumin (Rabbit) Specific Precipitates*

Horse No.	Bleeding date	Injection*	Antibody N per 5 ml.
			mg.
1046	Feb. 26, 1940	Globulin, i.v.	0.17
"	Apr. 8, 1940	" "	0.18
"	July 25, 1940	" "	0.19†
"	Oct. 21, 1940	End of rest period	0.11§
"	Dec. 23, 1940	Globulin, i.c.	0.10
"	Jan. 22, 1941	" "	0.07
"	Mar. 10, 1941	" "	0.11†
"	July 2, 1941	" s.c.	0.33†
"	Oct. 28, 1941	End of rest period	0.01†
"	Feb. 4, 1942	Albumin, i.v.	0.09§
1126	Feb. 4, 1942	Albumin-globulin, i.v.	0.14§
1127	Oct. 28, 1941	Before injection	0.00
"	Feb. 4, 1942	Albumin-globulin, s.c.	0.38§
999	Oct. 28, 1941	Before injection	0.00
"	Feb. 4, 1942	Globulin, s.c.	0.41§

\* i.v. = intravenously, i.c. = intracutaneously, s.c. = subcutaneously. Globulin, albumin, or albumin-globulin mixture used as indicated. Dosages given in Table I.

† Analyses on 4.0 ml. serum. All values recalculated to 5.0 ml. for comparison with other tables.

§ Analyses on 2.0 ml., recalculated to 5.

|| Analyses on 3.0 ml., recalculated to 5.

about 1.5 times as much as the antibody content per milliliter determined with the specific precipitate (Table IV), it is probable that the observed reaction was due to a minor component such as  $\beta$ -globulin present as an impurity in the  $\gamma$ -globulin antigen.

The bleeding of July 2, 1941, was also tested for low grade antibody which could not be precipitated directly by soluble antigens but which could attach itself to specific precipitates containing fully active, multivalent antibody (cf. 4, 11 to 13). Accordingly, duplicate 5 ml. portions of precipitating serum of horse 1046 (bleeding July 25, 1940, after intravenous injections and similar to that in Table III) were set up at 0° in three series: one with added saline, another with 1.0 ml. portions of the bleeding of July 2, 1941, and the third with 4 ml. portions of this bleeding. To each set of mixtures appropriate amounts of antigen were added. The determinations were carried out in the usual manner and are recorded in Table V. The

amounts of additional precipitate N due to the coprecipitation of antibody in the July 2, 1941, bleeding are given in the last column of the table.

The specific precipitate method was used to analyze the sera from the remaining horses injected subcutaneously with rabbit serum globulin (No. 999) or with mixed albumin and globulin (No. 1127). As noted in Table IV appreciable amounts of antibody were present. The direct precipitation reactions with  $\gamma$ -globulin were difficult to interpret, however, since large amounts of antigen were required and the precipitates obtained were relatively small.

*Fractionation of Antisera.*—In order to study the distribution of the various antibodies between water-soluble and water-insoluble fractions of the globulins of the antisera, 34 ml. of the concentrate of the serum of horse 1046 (Mar. 18, 1940) used in the experiment recorded

TABLE V

*Addition of Non-Precipitating Antibody from H 1046, Bleeding July 2, 1941, to Precipitates Formed from Rabbit  $\gamma$ -Globulin Antigen and Horse Serum H 1046, Bleeding July 25, 1940*

Bleeding July 25, 1940	Bleeding July 2, 1941	Antigen N added	Total N precipitated	Differences from blank
ml.	ml.	mg. 0°C. 3 days	mg.	mg.
5.0	1.0	0.013	0.132	0.009
5.0	4.0	0.013	0.138	0.015
5.0	0	0.013	0.123	—
5.0	1.0	0.039	0.240	0.034
5.0	4.0	0.039	0.272	0.066
5.0	0	0.039	0.206	—
5.0	1.0	0.129	0.334	0.082
5.0	4.0	0.139	0.486	0.234
5.0	0	0.129	0.252	—
5.0	1.0	0.387	0.322	0.044
5.0	4.0	0.387	0.410	0.132
5.0	0	0.387	0.278	—

in Table II were dialyzed against 3 daily changes of 400 ml. of 0.005 M phosphate buffer at pH 6.8. The precipitate (A) was centrifuged off and redissolved in saline. The solution gave an immediate precipitate with rabbit  $\gamma$ -globulin. The supernatant from precipitate (A), after addition of salt, was analyzed with a rabbit anti-egg albumin specific precipitate suspension and found to contain 44 per cent of the antibody originally present. By difference, 56 per cent of the antibody had been precipitated on dilution with water, a proportion somewhat lower than usual with the water-insoluble pneumococcus anticarbohydrate in the horse (14).

Another fractionation was carried out with a late bleeding (July 25, 1942) of horse 1127, which had received mixed albumin and globulin subcutaneously. The serum was precipitated with ammonium sulfate and the fractions coming down at  $\frac{1}{3}$  saturation, and between  $\frac{1}{3}$  and  $\frac{1}{2}$  saturation were each divided into water-insoluble and soluble fractions. The reaction of one of these (serum 1127 J,  $\frac{1}{3}$  to  $\frac{1}{2}$  saturated water-soluble) with rabbit serum albumin has been described in reference 2. The percentages of the total antibody recovered, as determined by analyses with an egg albumin anti-egg albumin specific precipitate, were: from the water-



insoluble portion of the fraction precipitated by  $\frac{1}{3}$  saturation with ammonium sulfate, 13 per cent; from the water-soluble portion, 23 per cent; from the water-insoluble portion of the fraction precipitating between  $\frac{1}{3}$  and  $\frac{1}{2}$  saturation, 2 per cent; from the water-soluble portion, 62 per cent. The water-soluble antibodies, which, in other experiments (2), reacted with soluble rabbit albumin as do antitoxins with toxins, comprised 85 per cent of the total.

#### DISCUSSION

The production of antibacterial (anticarbohydrate) antibodies by the intravenous injection of horses has been shown to be correlated in most instances with an increase in the amount of electrophoretic  $\gamma$ -globulin (15, 16), with occasional instances in which pneumococcus anticarbohydrate occurred in a new component ( $\beta_2$  or T) with mobility between those of the  $\beta$ - and  $\gamma$ -globulins (6, 16, 17). On the other hand, antitoxin produced in the horse occurs almost exclusively in this new fraction, absent in most normal horse sera (18, 19).

The electrophoretic patterns obtained in the present series of studies are, in general, those to be expected from the earlier work quoted. No indication is found in the patterns obtained with the sera of either horse 1046 or 1126 of the formation of a new component with mobility between the  $\beta$ - and  $\gamma$ -components. These horses received intravenous injections. In contrast, the patterns for sera 999 and 1127 clearly showed the formation of a new  $\beta_2$  or T component after subcutaneous injections of rabbit serum albumin. The antigens and the injection schedules for horses 1126 and 1127 were identical; only the routes of injection were varied (Table I), horse 1126 having been injected intravenously, horse 1127 subcutaneously with the same albumin-globulin mixture (*cf.* (2)).

The antibody present in the serum of horse 1046 after 4 months of intravenous injections with rabbit globulin gave a typical precipitin reaction with a soluble antigen—normal rabbit electrophoretic  $\gamma$ -globulin. This reaction (Fig. 1) is of the type given by pneumococcus anticarbohydrate (20) and anti-protein (3) in the horse. Characteristic is the absence of a prezone in the region of antibody excess; instead, the curves may be extrapolated to the origin. This is, of course, in marked contrast to the behavior of the rabbit serum albumin—horse anti-albumin system (2), and other examples of the so called flocculation reaction (11, 19, 21, and 22).

The data for the globulin anti-globulin system are best represented by an empirical equation (Table II) involving the first and the  $3/2$  power of the quantity of antigen added and precipitated, as first proposed for several other precipitating systems involving antiprotein formed in the rabbit (23, 4, 8, 9). Although this equation has not yet been derived from fundamental considerations, as has another which best represents numerous other systems (5*b*, 20*d*) it has the merit in these instances of fitting linearly a plot representing the ratio of antibody N: antigen N precipitated against the square root of the amount of antigen N added. For comparison of different sera, the data are recalculated to a common antibody content, for example, 1.0 mg. N per ml.

When the bleeding taken from horse 1046 after a 7.5 month intravenous

course was set up with rabbit  $\gamma$ -globulin at two temperatures,  $0^\circ$  and  $37^\circ$  (Table III), a rather marked variation of reactivity with temperature was noted. The antibody precipitable at  $37^\circ$  was only 0.123/0.174, or 71 per cent of that removed at  $0^\circ$ . This resembles closely the findings obtained with anticarbohydrate systems in the horse (20 c) and in the rabbit (24). On the other hand, precipitating antiprotein (anti-egg albumin) in the rabbit (4) or flocculating antiprotein systems in horse sera (rabbit serum albumin (2); egg albumin (11, 12); diphtheria toxin (21)) have practically negligible temperature coefficients.

The maximum combining ratio (20 d, 4) at  $37^\circ$ —obtained by extrapolation to zero antigen N of the line giving the variation of the ratio antibody N: antigen N precipitated with antigen N added—is also less than at  $0^\circ$ . At the higher temperature it is calculated that only 8.6 mg. of antibody N can be removed per mg. of  $\gamma$ -globulin N, compared with 14.4 mg. at  $0^\circ$ .

As shown in Table IV, the total amount of antibody to  $\gamma$ -globulin, determined at  $0^\circ$ , increased very little (0.18 to 0.20 mg. N/5 ml.) in test samples of the serum of horse 1046 during the last 3 months of the intravenous injections. The quantitative properties of the antibodies show appreciable differences, however. Since the data for Table II were obtained on a dilution of a globulin solution, while those for Table III were on whole serum of different antibody content, it is necessary to compare them on some common basis, such as 1.0 mg. of precipitable antibody N. When this is done the following equations are obtained:

	Bleeding date 1940	Mg. antibody N precipitated =
(1)	Mar. 18	9.3 ( $\gamma$ G N) minus 10.9 ( $\gamma$ G N) <sup>1/2</sup>
(2)	July 8	16.3 ( $\gamma$ G N) minus 26 ( $\gamma$ G N) <sup>1/2</sup>
(3)	July 25	14.4 ( $\gamma$ G N) minus 21 ( $\gamma$ G N) <sup>1/2</sup>

It is evident from the above that the initial combining ratio (9.3) and the slope (10.9) characteristic of the March 18, 1940, bleeding (equation (1)) are both significantly lower than the corresponding constants for the later bleedings (equations (2) and 3)). The changes in these two factors are in accord with other quantitative data (4, 12) and with general experience that the reactivities of antibody frequently tend to broaden on progressive immunization.

It will be noted from Table IV that the antibody concentration in the serum of horse 1046 dropped from 0.18 to 0.10 mg. N per 5 ml. at the end of the rest period following intravenous injection of globulin. The antibody content remained practically constant after a series of intracutaneous injections (bleeding March 10, 1941) and then increased markedly after a further course of subcutaneous injections to 0.33 mg./5 ml. (July 2, 1941). This increase, however, was due to the gradual replacement of the precipitating antibody by "univalent" antibody (4, 11, 12) which did not precipitate with soluble antigen. The value given was obtained by addition to the serum of a washed specific precipitate composed of egg albumin and rabbit anti-egg albumin. This

antibody has been shown to be in the  $\gamma$ -globulin fraction (6) of rabbit sera. This device consequently permitted the use of rabbit  $\gamma$ -globulin in an insoluble form with which the "univalent" antibody could combine and be measured quantitatively.

That the precipitating or "multivalent" form of antibody should not recur during the intracutaneous and subcutaneous injections subsequent to the rest period was indeed unexpected, especially since new antibody of low grade or "univalent" reactivity was produced. Mixtures in various proportions of earlier precipitating bleedings with the non-precipitating antibodies actually gave precipitates with soluble  $\gamma$ -globulin (Table V), providing evidence against any markedly inhibitory action of the "univalent" antibody which might mask the presence of a small amount of residual precipitating antibody. Failure of the precipitating antibody to reappear when the route of injection was changed points strongly toward the essential independence of the physiological mechanisms for producing the two forms of antibody.

When the antibody is removed by attachment to preformed precipitates (egg albumin anti-egg albumin) (Table VI), lower values of the constants are obtained than in Table III, possibly because only the rabbit  $\gamma$ -globulin molecules at the surfaces of the particles are available for interaction with the anti-globulin in the horse serum, or perhaps because of masking of portions of the rabbit globulin configuration by the egg albumin.

While most of the antibodies formed in horses after the subcutaneous injection of rabbit serum albumin are to be found in the water-soluble fraction of the globulins of the antisera (2), the antibodies developed in response to the intravenous injection of rabbit globulin are largely water-insoluble. The quantitative reaction curves (Tables II and III) are also similar to those obtained with bacterial carbohydrate-anticarbohydrate systems in horse sera (20 c, d) and show the same marked temperature coefficient. The antibody formed in response to subcutaneous injection of globulin differs most strikingly from that produced after intravenous injection in its failure to precipitate with globulin in solution.

Since it has now been amply shown that the zonal type of flocculation is not the only type of reactivity possible in antiprotein systems in horse sera the older classification into anticarbohydrate and antiprotein reactions therefore appears to be an oversimplification. According to Kendall (25) the differences in reactivity between the precipitin type of antibody and the zonally flocculating antitoxin can be accounted for quantitatively by the assumption that in the former molecule two groups reactive with antigen (bivalent antibody) are alike, and that in the antitoxin molecule the two groups differ in affinity.

Another instance is also provided of the occurrence of low grade antibody free from the precipitating form with which it usually occurs. Horse anti-egg albumin with this property has previously been described (11, 12) as occurring

in an early stage of the immunization, while serum from later bleedings gave a characteristic zone of flocculation.

It is accordingly clear that the horse can produce a number of antibodies with differing chemical, physical, and serological properties. The route of injection and the nature of the antigen are major factors in determining the type of re-

TABLE VI

*Absorption of Antibodies to Rabbit Globulin in Sera of Horse 1016 by Means of a Specific Precipitate*

Per 5.0 ml. horse serum H 1016, 0°C., 3 days

Egg albumin rabbit anti-egg albumin suspension N added	Bleeding July 25, 1940, visibly reactive with a soluble antigen (as in Table III)			Bleeding July 2, 1941, non-reactive with a soluble antigen (see text, pp. 99, 100)		
	Total N precipitated	Antibody N (by difference)	Antibody N Suspension N	Total N precipitated	Antibody N precipitated (by difference)	Antibody N Suspension N
mg.	mg.	mg.		mg.	mg.	
0.106*	0.203	0.097	0.92	0.203	0.097	0.92
0.213*	0.370	0.157	0.74	0.377	0.164	0.77
0.283‡	0.455	0.172	0.61	0.475	0.192	0.68
0.425	0.606	0.181	0.43	0.692	0.267	0.63
0.638	0.834	0.196	0.31	0.918§	0.280	0.44

Reaction equations||

Mg. antibody N precipitated =  $1.43 (S N) - 1.54 (S N)^{3/2}$ . A maximum (calculated) = 0.18 mg. N

Mg. antibody N precipitated =  $1.26 (S N) - 1.04 (S N)^{3/2}$ . A maximum (calculated) = 0.27 mg. N

Conversion to basis of 1.0 mg. maximum precipitable antibody N, A, gives  
Mg. antibody N precipitated =  $1.43 (S N) - 0.65 (S N)^{3/2}$

to 1.0 mg. A N:  
Mg. antibody N precipitated =  $1.26 (S N) - 0.54 (S N)^{3/2}$

A = antibody.

\* Double quantities of antigen and serum actually used.

‡ One and one-half quantities actually used.

§ One determination only.

|| S N = antigen suspension N added.

sponse. Rabbit serum albumin does not appear to be antigenic in the horse when administered intravenously but leads to the formation of the antitoxic type of antibody when given subcutaneously. Rabbit serum globulin, on the other hand, functions as an antigen by both routes, but stimulates the production of precipitating antibodies only when injected intravenously.

It is not clear what property of the antigen might be concerned in these effects. Egg albumin, serum albumin, and diphtheria toxin are of lower molecular weight than serum globulin, which produces precipitating antibodies in

the horse, but molecular size cannot be the sole decisive factor since the subcutaneous injection of horses with hemocyanin (molecular weight 7,000,000) results in antibody of the antitoxin type (26). Clarification of this problem must therefore await further study.

#### SUMMARY

1. The intravenous injection of two horses with alum-precipitated rabbit serum globulin resulted in the production of antibody which gave a typical precipitin reaction without a prezone in the region of antibody excess.

2. The chemical, physical, and serological properties of this antibody are comparable to those of the more familiar anticarbohydrate antibodies.

3. The subcutaneous injection of horses with the globulin antigen gave rise to low grade "univalent" antibody which did not precipitate with soluble antigen.

4. The low grade antibody could be removed from solution by attachment to preformed specific precipitates, or by coprecipitation in the presence of "multivalent" precipitating antibody.

5. It is concluded that the familiar antitoxin type of antibody is not the only form of antiprotein response in horses but that precipitating and low grade non-precipitating antibodies may also be formed.

6. The nature of the antigen and the route of injection are demonstrated to be important factors in determining the characteristics of the antibody formed.

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## ON THE ORIGIN OF HEPARIN

### AN EXAMINATION OF THE HEPARIN CONTENT AND THE SPECIFIC CYTOPLASMIC PARTICLES OF NEOPLASTIC MAST CELLS

By JEAN OLIVER, M.D., FRANK BLOOM, D.V.M., AND CARMEN MANGIERI\*

*(From the Department of Pathology of the Long Island College of Medicine,  
Brooklyn, and the Laboratories of Hoffman-La Roche, Nutley)*

PLATES 6 TO 9

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Tumors arising from a tissue that normally produces substances which affect generally the biological activity of the organism present a double interest to the investigator. Not only can they be studied from the viewpoint of the neoplastic process, but there remains the concomitant problem of the disturbances produced by this process in the formation of the biologically active substances and the consequent widespread effects that may result from these alterations. The latter, as with tumors of the parathyroid or the gonads, are usually plainly evident, but the changes, whether quantitative or qualitative, in the nature and formation of the active substances remain obscure.

In the present investigation a tumor is examined in which alterations in a biologically potent substance can be examined by the techniques of morphology, biochemistry, and histochemistry. A correlation of these varied data with the functional activity of the tumor tissue will be attempted.

Mast cell tumors, first described by Bloom (1), are a not uncommon neoplasm in dogs and so present an exceptional opportunity for the study of these cells of obscure origin and uncertain function. This is true, not only because great numbers of mast cells are available in the tumor for study in almost "pure culture," but also, since the tumors vary greatly in those growth characteristics which may be summarized as "malignancy," because they present a correspondingly varying degree of anaplasia in their constituent cells. The opportunity is thus afforded for the examination of mast cells of every degree of maturity from primitive forms with sparse fine granulations to mature cells crowded with their coarse metachromatic granules. It is in particular this cellular variation that is to be used in our study as a means to examine one aspect of their function, namely their rôle in heparin formation.

Following Jorpes's (2) demonstrations of the metachromatic reaction of heparin and of its chemical constitution as a member of the group of mucoitin-sulfuric acid esters, Wilander (3) showed the correlation that exists between the variation in frequency of mast cells in the liver capsule and the liver parenchyma of the ox and the varying content of the two tissues in heparin. The latter was

\*Morphological, chemical, and clinical observations by J.O. and F.B., biological assays by C.M.

prepared by the method of Charles and Scott (4) and the amount of heparin found expressed in the conventional unit content that measures its ability to prevent coagulation of blood. Ten times as much heparin was found in the liver capsule where mast cells are frequent as in the parenchyma where they are present in moderate numbers. No heparin was obtained from the liver of rats which contains no mast cells, while in the subcutaneous tissue of the rat, with its moderate mast cell content, as much was found as in the ox liver.

Our problem is in its first part similar to that of the investigators just mentioned; to determine the heparin content of the tissues of mast cell tumors. Since the quantity of mast cells greatly surpasses that in any normal tissue, the heparin content of the tumor tissue should greatly exceed that obtainable from any normal tissue. This conclusion presumes that the tumor mast cells maintain their usual function, a presupposition supported by what is known of tumors of other analogous secretory tissues, such as the parathyroid or the pancreatic islets and gonads. As our findings will show, not only is this supposition confirmed, but by the examination of two mast cell tumors of varying degrees of cellular maturity an interesting correlation between function and structure of the tumor cell becomes apparent that casts further light on the ultimate origin of heparin.

### *The Tumor Material*

Since the original description (1) of the mast cell tumor of the skin of dogs, 15 additional cases have been observed. These neoplasms occur most commonly in older animals and males are more often affected. The tumors may be either solitary, in which case they are usually benign, or multiple and malignant. The gross appearances of the tumors in different animals vary considerably as concerns their size and number and the extent of superficial ulceration that ultimately occurs. In the skin, the tumors are subepithelial and occur principally on the trunk, with less common involvement of the extremities. Metastatic lesions, either microscopic or macroscopic, commonly occur in the spleen, liver, and regional lymph nodes. The general health of the dogs is usually little affected except in the terminal stages of the malignant forms when anemia, cachexia, and other symptoms manifest themselves. No abnormalities in the clotting time of the blood have been observed. Repeated attempts to transmit the tumors to other dogs have been unsuccessful.

The tumors consist essentially of atypical tissue mast cells whose cytoplasm contains metachromatic basophilic granules. The granule structure is best demonstrated by staining tumor imprints with Wright's stain. As has been mentioned, the morphologic characteristics of the mast cells in the various tumors show all gradations from mature cells that resemble the normal tissue mast cells to immature cells that manifest various degrees of anaplasia. While it is generally true that the mature cells have large coarse granules and that the immature cells have finer granules, exceptions occur inasmuch as cells with



atypical nuclei may have coarse granules and cells otherwise quite mature in appearance may have fine granules. All combinations of cellular maturity and immaturity with fine and coarse granules have been found in the series of tumors examined.

The general structure of the tumor consists of mast cells heavily infiltrating the corium and subcutaneous tissue. The cells may be grouped in nodular collections or form diffuse sheet-like infiltrations. The metastatic lesions resemble in their cellular content the primary tumor. Invasion of blood vessels is frequently seen and the sinuses of the spleen may be filled with tumor cells.

### *I. The Heparin Content of a Mast Cell Tumor of Mature Cell Type*

A 10 year old male Pointer had for 1 year a spherical nodule 2.7 cm. in diameter in the skin of the scrotum. The nodule was elevated 6 mm. above the surrounding skin and the surface was superficially ulcerated. Under local anesthesia, the entire scrotum and testes were removed. Ten weeks following the operation, examination revealed a round firm swelling that measured 10 cm. in diameter in the region of the left peripenile lymph node. The skin over the enlargement and for an area of 15 cm. in diameter in the left inguinal region and the inner surface of the thigh was diffusely thickened to a depth of 6 to 12 mm. The involved skin was corrugated and pale tan in color. In consideration of the extent of the tumor process, the dog was killed with a lethal dose of soluble pentobarbital given intravenously. Necropsy showed no outstanding gross lesions with the exception of enlargement of the right peripenile lymph node which measured 2.6 by 3.7 cm. and replacement of the sublumbar lymph nodes with tumor tissue. The latter nodes were 3 in number and were greatly increased in size. The largest measured  $6 \times 9$  cm. and the smallest measured  $3.5 \times 5$  cm.

For histological examination tissues from the subcutaneous tumor and also from regional lymph nodes and viscera were fixed in Zenker's fluid and sections were stained with the Giemsa method and toluidine blue. Imprints were also made of all the tissues and stained immediately with Wright stain. The detailed cytological studies were made on these latter preparations as all danger of solution of granules was thus avoided.

### *Cellular Structure of the Tumor*

Sections showed the epidermis either intact and thinly stretched over masses of tumor or ulcerated by its extension to the surface. In the corium and extending deep into the subcutaneous tissue were solid infiltrations of mast cells (Fig. 1). These cells were of mature type, resembling in all details the normal tissue mast cell except perhaps for their somewhat larger size. They were so filled with metachromatic granules that in most instances all cellular detail was obscured, even the nucleus being covered by the gross granules (Fig. 2). When visible the nucleus was of moderate size, oval, with finely divided chromatin and obscure nucleoli. The mast cells lay in a stroma of collagen fibrils which varied inversely in density with the concentration of the tumor cells; in no areas was there extensive fibrosis. In spite of the lack of cellular anaplasia, the tumor was definitely malignant as was evident not only from local invasion but by the occurrence of metastases in lymph glands, spleen, and liver. The sinuses of the pulp of the spleen and branches of the portal vein were filled with solid masses of similar mature tumor mast cells.

In the imprints of the tumor the mast cells,—since they were freed of tissue pressure and lay in tissue fluid while the slide was being pressed upon the fresh cut surface,—had lost their polygonal or irregular shape and assumed spherical contours (Fig. 4). Except for this difference in shape the cells showed the same structural characteristics as in sections. The granules, however, were better preserved and the details of their shape and size were more apparent. The cytoplasm of all the cells was filled with them, some being so crowded that no detail of cell structure could be seen. The granules varied in shape; some were spherical, others ellipsoid, and a few presented the appearance of short rods. Many lay free in the tissue fluid between the cells. They were strongly metachromatic when stained with toluidine blue or with Wright's stain, whereas the nuclei stained a contrasting blue with the latter method.

In summary, the tumor consisted of a mass of mast cells that morphologically appeared identical with the coarsely granular tissue mast cells of the normal subcutaneous tissues.

#### *The Extraction of Heparin*

330 gm. of subcutaneous tumor tissue were emulsified in a Waring blender and made up to 3300 cc. in phosphate buffer at a pH of 6.8. Toluol was added to the mixture and it was then autolyzed at 37°C. for 24 hours. The alkalinity was raised with NaOH to pH 9.0 and extraction continued for 30 minutes at 70°C. The emulsion was then made acid with glacial acetic to a pH 4.5 and heated for 10 minutes at 85°C. A heavy coagulation of proteins occurred which was removed by filtration. A clear opalescent filtrate resulted. After standing 12 hours in the ice box a further slight precipitate occurred which was removed by filtration, and to the clear filtrate 3 volumes of absolute alcohol were added. A heavy white precipitate resulted which after 12 hours in the ice box was removed by centrifugation. The precipitate was washed twice in alcohol, dried in a desiccator, and found to weigh 20.1 gm.

Although a preparation procured in the way described represents an extremely crude "heparin" and must contain a large component of proteins and glycoproteins other than heparin (5) it was decided to assay its biological activity at this point rather than to risk the losses that would accompany its purification. The method devised by one of us was used (6). The assay showed, with an optimum dosage of 60  $\gamma$  per cc., an activity of  $\pm 1$  A.C.U. (8.2 I.U.)<sup>1</sup> per mg. With increasing dosage the titre fell; 100  $\gamma$  per cc. equaling 5.5 A.C.U. (1 I.U.) per mg. This decrease in titre when an excessive amount of the preparation was used in the titration was considered to be the effect of contaminating protein and thrombokinase. Jaques and Charles (7) have discussed these difficulties in the assay of crude preparations and have pointed out that when the anticoagulant activity is less than 5 I.U. per mg. it may become necessary to decide whether the activity is due to heparin or other substances. Our preparation lay just above this minimum figure so that it was decided to attempt some purification to raise the activity per milligram to a figure that would

<sup>1</sup> A.C.U., anticoagulating unit; I. U., international unit.

be incompatible with any other assumption than that it was due to heparin. A modification of the first step in the method of purification of Charles and Scott (4) was used. The product of their complete procedure is still considered by these authors to be "crude heparin."

2.5 gm. of the preparation was dissolved in 100 cc. of  $H_2O$  at a pH of 8.5 and digested with 2.0 gm. of pancreatin for 36 hours with xylene at  $37^\circ C$ . 2 volumes of 95 per cent alcohol and 0.5 cc. of HCl were then added. A light precipitate occurred and after 12 hours in the ice box was removed by centrifugation. It was dissolved in 50 cc. of  $H_2O$  at pH 8 and heated to  $75^\circ C$ . A small amount of dark material was removed with the centrifuge and to the clear supernatant 2 volumes of acetone and 0.3 cc. HCl were added. The flocculent precipitate was removed, washed in alcohol, and dried. Only 0.080 gm. was recovered, but this product on assay showed an activity of 147 A.C.U. (29.4 I.U.) per mg. This is an increase in potency of  $3\frac{1}{2}$  times over the original preparation and is compatible with the conclusion that the anticoagulant effect is due to heparin.

In estimating the total heparin content of the tumor tissue the assay of the first sample was used, since much had been lost in the attempt at purification. Since 1 mg. of this preparation contained 41 A.C.U. and 20 gm. of preparation was obtained from 330 gm. of tumor, a kilo of tumor would contain 2,460,000 A.C.U. or 492,000 I.U. Dog's liver contains about 10,000 I.U. per kilo so it follows that the mast cell tumor contained almost 50 times as much heparin as has been obtained from the richest normal source of the most active form of heparin.

## *II. The Heparin Content of a Mast Cell Tumor of Immature (Anaplastic) Cell Type*

The tumor was situated on the right chest of an 8 year old male fox-terrier. Two previous attempts at surgical removal had been unsuccessful for the tumor recurred in each instance several months following operation. On examination, the tumor was 18 cm. in diameter and elevated 4.5 cm. above the surface of the skin. The covering epithelium was intact with the exception of an ulcerated area 3.6 cm. in diameter on the upper edge of the tumor. In the immediate vicinity of the main tumor mass were 5 small subepithelial nodules that varied from 0.8 to 1.7 cm. in diameter. Under general anesthesia, the entire tumor tissue including the small nodules was surgically removed. On section, the tissue was a deep grey color and firm to palpation. This material was used for heparin determination. The operative wound healed uneventfully and the animal was observed at frequent intervals. No evidence of recurrence was noticed at these examinations. Three months following the last operation, the animal died of a strangulated perineal hernia. Necropsy, both macroscopic and microscopic, showed no lesions pertinent to the history of the tumor.

### *The Cellular Nature of the Tumor*

Histological sections of the subcutaneous tumor showed masses of tumor cells lying beneath the epidermis and invading the deeper tissue. Even with low magnification a striking difference from the first specimen could be noticed in the appearance of the tumor. The tumor cells were less clearly seen, for their abundant cytoplasm appeared relatively pale as compared to the densely

stained granulations of the first tumor (Fig. 3). This contrast in cytological detail was most apparent, however, in the imprint preparations (Fig. 5). In these the cells varied greatly in size, some having twice the average diameter of those of the previous tumor. The abundant cytoplasm in every case contained granules, but these varied from dust-like particles to the typical coarse metachromatic granules of the mature cell. The latter were at best, however, few in number and widely scattered and in no instance was the nucleus obscured by them. The nucleus was spherical or oval, vesicular with fine scattered chromatin, and one or two large nucleoli were usually present. With Wright's stain it took on the definite reddish tone of metachromasia appearing very unlike the blue nucleus of the more mature cells of the first tumor. These contrasts between the morphological maturity of the heavily granular cells of the first tumor and that of the lightly staining anaplastic cells of the second tumor will be best appreciated in the comparison of Figs. 4 and 5.

To summarize, the tumor consists of anaplastic mast cells of immature type, the more primitive forms resembling very closely, with their large nuclei and fine granulation, the early stages in the development of tissue mast cells described by Downey (8). In occasional cells the typical coarse metachromatic granules of the adult form were sparsely present.

### *The Extraction of Heparin*

168 gm. of subcutaneous tumor was ground in a Waring blender and submitted to the same procedure as the previous material: autolysis at 37°C. at pH 6.8, extraction at 70°C. at a pH of 9 in phosphate buffer, filtration, acidification with acetic acid to a pH of 4.5, coagulation of proteins at 65°C., filtration, and final precipitation with 3 volumes of alcohol. A dried precipitate of 9.05 gm. was obtained.

As it was suspected that the heparin yield in this tumor might not be so abundant as in the previous experiment, it was decided to repeat, at a higher degree of alkalinity, the extraction of the usually discarded first precipitation of proteins. This precipitate was therefore re-suspended in 250 cc. of 0.5 NaOH and heated to 70°C. for 30 minutes. It was then acidified to pH 4.5 with acetic acid and reheated to 70°C. The protein precipitate was removed by filtration and to the clear supernatant 3 volumes of alcohol were added. The resulting precipitate, after washing in alcohol and drying, weighed 1.50 gm.

Assays of the two preparations obtained as described showed that the first sample extracted with the relatively weakly alkaline phosphate buffer had no determinable anticoagulant effect. The second sample extracted by stronger alkali, showed an anticoagulant activity of 9.6 A.C.U. (1.9 I.U.) per mg.

If one accepts this weakly anticoagulant effect as due to the presence of heparin the content per kilo of the latter would be 86,400 A.C.U. or 17,280 I.U., since 1.50 gm. was obtained from 168 gm. of tumor. On this assumption the immature tumor therefore contained only about one twenty-ninth as much heparin as the mature tumor but nevertheless 1.7 times as much as does normal dog liver (7).

*The Particulate Content of Living Neoplastic Mast Cells*

In preparations fixed and stained with appropriate methods the mature cells of the tumor rich in heparin were seen to be filled with coarse metachromatic granules, while in the immature cells of the tumor of moderate heparin content the granules were present as fine dust-like particles. Whether there were as many of these fine granulations in the cell protoplasm as there were of the coarse granules in the mature cells is impossible to determine, since their minuteness faded into invisibility in the stained preparation.

Phase contrast microscopy allows the examination of living cells unaltered by reagents so this method was applied to the examination of the cytoplasmic particulate material of the tumor mast cells.<sup>2</sup>

The cells were prepared for examination by scraping the surface of the fresh tumor and mounting the resulting turbid fluid in the usual manner of a fresh preparation beneath coverslip and slide. One of the tumors selected resembled the mature tumor of rich heparin content while in the other the anaplastic cells of an immature tumor contained a mixture of fine and moderately coarse particles.

Figs. 6 to 11 show living unstained cells from an immature tumor. Detail is best seen in Fig. 6, as in this specimen gentle pressure on the coverglass had flattened the cell without disrupting its content; in the remaining figures the cells were floating in fluid and therefore their thickness disturbed somewhat the clarity of the phase contrasts.

In Fig. 6 three optical effects are produced by the particulate matter of the cytoplasm; this appears to be composed of densely black granules, similar dark granules with optically clear centers, and fine, ill-defined grey particulate material. The dark granules appear in irregular clusters scattered on a diffuse background of the grey material. In the interpretation of these optical effects it is to be remembered that with phase contrast microscopy the examination is made with diaphragms widely open and that contrasts are at a maximum only when the objects are in exact focus, so that the differences in the optical appearance of the various granules in these figures are not due to the diffraction artifacts that result from "cutting down the light" and "out of focus" examination that are so commonly used in conventional microscopy to emphasize a detail which is largely spurious. We can therefore be certain that the photographed differences in the particulate matter are at least optically real.

Fig. 12 shows a cell from a mature tumor the cells of which are filled with coarse metachromatic granules similar in stained preparations to those shown in the cell in the upper left hand corner of Fig. 4. It will be seen that the cell appears distended almost to bursting with dark granules of even greater size than those of the anaplastic cells and that scattered among them are a few of

<sup>2</sup> The Zeiss apparatus for phase microscopy was kindly loaned by Dr. Hans Zollinger of the University of Zurich.

the indefinite grey particulate bodies. The picture is therefore the exact converse of the immature cell of Fig. 6. Figs. 7 to 11 show cells with varying amounts of the two forms of particulate matter.

#### DISCUSSION

If the mast cell seemed proven to be the normal source of heparin by Wilander's (3) correlation of the concentration of these cells in normal tissues with their heparin content, then this conclusion is not merely confirmed but is pushed close to its logical limit of certainty by our demonstration of the massive heparin content of a tumor composed of well differentiated mast cells. It is also evident from our findings that the heparin content of mast cell tumors may vary with the granule content of the tumor cells in not only a quantitative but also a qualitative sense. In the anaplastic mast cells of an immature tumor granules were present and numerous, but their fine dust-like character in stained preparations contrasted strikingly with the coarse granule of the mature cell. In such a tumor the heparin content was relatively low.

Since the granule of the typical mast cell is strongly metachromatic, a property also of the heparin which can be extracted from it, the assumption has been general that the coarse granule is heparin in particulate form. Our demonstration of the variation in the heparin content of the tumor with the variation in the structure as well as number of the granules reopens this question and allows an examination of an earlier phase of the functional activity of the cells. For Downey (8) in his studies of the histogenesis of the normal tissue mast cell has traced back the coarse metachromatic granules of the adult cell to earlier fine dust-like particles which are not metachromatic. This dust-like material he derives ultimately from the nucleus. Our findings with anaplastic mast cells afford no conclusive evidence on this point though the contrast between the reddish metachromasia of the nuclei of the immature cells and the blue nuclei of the mature might be considered indicative of a passage of metachromatic material from nucleus to cytoplasm.

The examination by means of phase contrast microscopy of living tumor mast cells unaffected by fixative or stain clarifies considerably the uncertain data of the stained preparations. Under living conditions the cytoplasmic particulate matter appears in two forms; as an indefinite grey granular material and as densely black discrete granules. The former is the dust-like material of the stained immature cell and the latter are the coarse metachromatic granules of the stained adult cell. A comparison of Fig. 6 showing an immature cell with scanty dark granules and rich content of grey granular material with the mature cell of Fig. 12 in which the greyish particulate matter is virtually absent but dense black granules are present instead leaves little doubt that the morphological expression of the maturation of these tumor cells involves either a replacement of the greyish particulate matter by granules which appear dense

and black or a transformation of the former material into a substance having the latter character. Our biochemical determinations have shown that the heparin content of the immature tumor cell is comparatively small. It is therefore possible in a series of mast cell tumors to correlate the formation of heparin with the maturing of the primitive granular material of the anaplastic cell and its replacement by, or transformation into, the strongly metachromatic coarse granules of the more mature tumor type, with its abundant heparin content. There would seem to be, therefore, a primitive particulate material in the mast cell in which "heparin" formation, or accumulation, has not yet occurred.

These conclusions concerning the structure and function of the tumor mast cells are supported by the results of tissue culture, which are reported in an accompanying paper (9). Briefly, pure cultures of granular mast cells grew from explants made of tissue from the immature second tumor of the present study. In the growing preparations gradation of granule size was not apparent, but variation in the metachromasia of the granules in the proliferating mast cells was clearly evident. In some cells metachromasia was intense and in others it was only feebly present, while in still others both metachromatic and unstained granules were present in mixture (reference 9, fig. 3). These experimental findings, along with our demonstrations of the varied heparin content of mast cell tumors, also direct attention to the possible existence of a pre-heparin primitive particulate matter as a structural precursor in the genesis of the functionally active product of the cells.

A final question concerns the qualitative nature of the heparin of the mast cell tumors. Jaques, Waters, and Charles (10) have shown that crystalline barium salts of heparin from various species show variation in their biological activity, though no chemical difference was noted between the different samples. Dog heparin for example is 10 times as potent per mg. as that derived from sheep tissue. One would like to know whether the heparin from a dog tumor is identical with that derived from normal dog tissue. In our calculations of "heparin content" only biological activity was measured; if tumor heparin is more potent than heparin of normal tissue then the actual heparin content of the tumors we examined was somewhat less than 50 times that of dog liver, whereas if the tumor heparin is less potent, the actual content must have exceeded the figure given.

Of even greater ultimate interest is the possibility that the heparin from neoplasms may differ in its chemical constitution from that derived from mature normal tissues, representing perhaps a precursor in the formation of the normal substance or a variant on its constitution. In the case of the mature tumor, from which active "heparin" was obtained in such large amount by chemical manipulation, great numbers of mast cells with mature granulations were present in the portal blood stream and in the sinuses of the spleen, yet no anticoagulative activity was observed *in vivo*. Such a paradox empha-

sizes the present uncertainty not only as to the exact chemical structure of isolated heparin but in particular as to the form in which it occurs in nature. A more detailed biochemical examination of the heparin of tumors is therefore indicated.

#### SUMMARY AND CONCLUSIONS

1. The spontaneous mast cell tumor of the dog contains heparin.
2. The cytoplasmic particulate content of the tumor mast cells varies with their anaplasia. This conclusion is based on the following findings: (a) in the immature cell of the more malignant tumor the particulate matter appeared in the living cells by phase microscopy to be composed of greyish ill-defined particles or as a fine, weakly metachromatic granulation in the fixed and stained preparation; (b) in the mature cells of a relatively benign mast cell tumor, both in the living cell and in stained preparations, the particulate matter occurred in the form of discrete, dense, and strongly metachromatic granules, resembling those of the normal mast cell.
3. The heparin content was large (fifty times that of dog liver) in the growth with mature cells and only moderate (1.7 times) in that with immature cells.
4. Since there may be a great amount of greyish particulate matter (or fine stained granules) in a tumor of relatively low heparin content, it is suggested that this material represents an early or precursor phase in the development of heparin.
5. This possibility and the fact that the blood stream may be invaded by mature tumor mast cells of large heparin content without evident disturbance in the coagulability of the blood suggest the value of a comprehensive biochemical study of the heparin of mast cell tumors.

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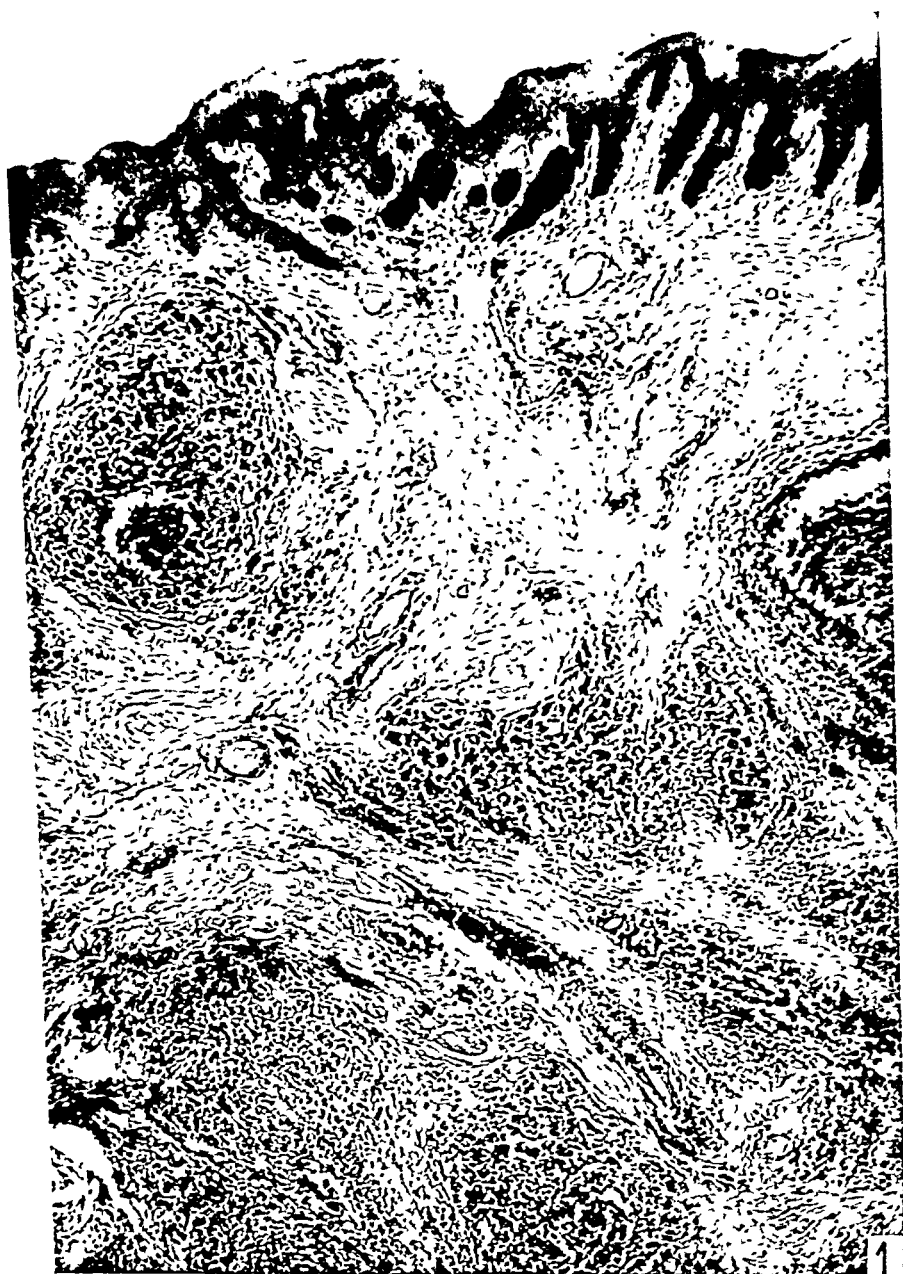
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#### EXPLANATION OF PLATES

##### PLATE 6

FIG. 1. Mast cell tumor of mature cell type described in the text. Beneath the epidermis and invading the deep fibrous subcutaneous tissues are seen dense infiltrations of tumor mast cells. Dominici stain.  $\times 70$ .

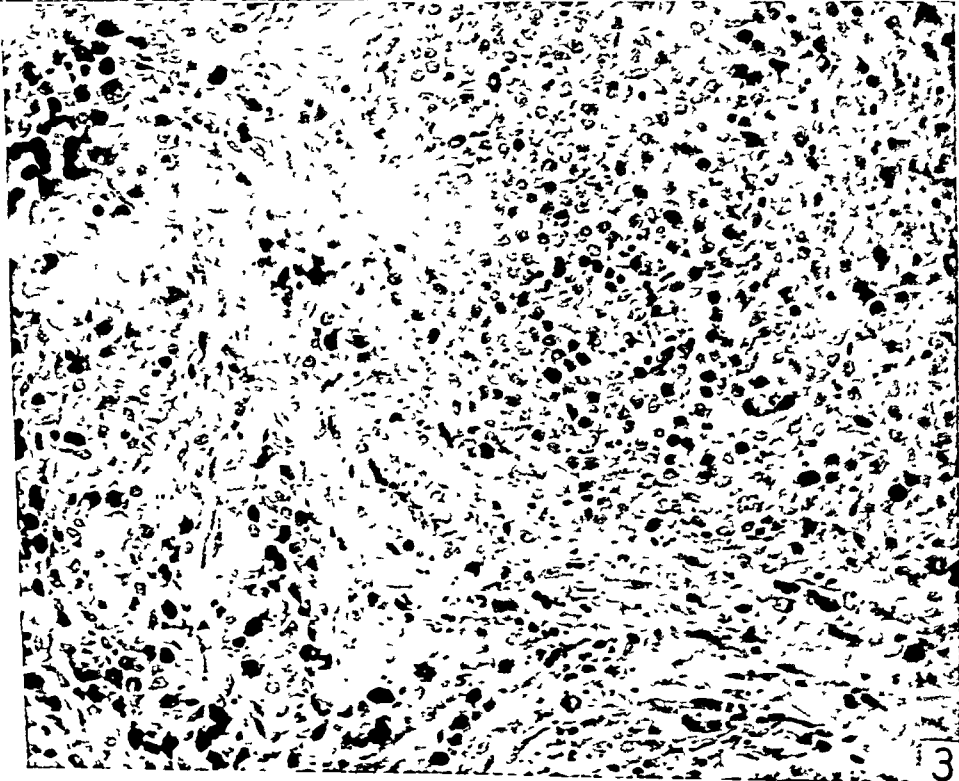
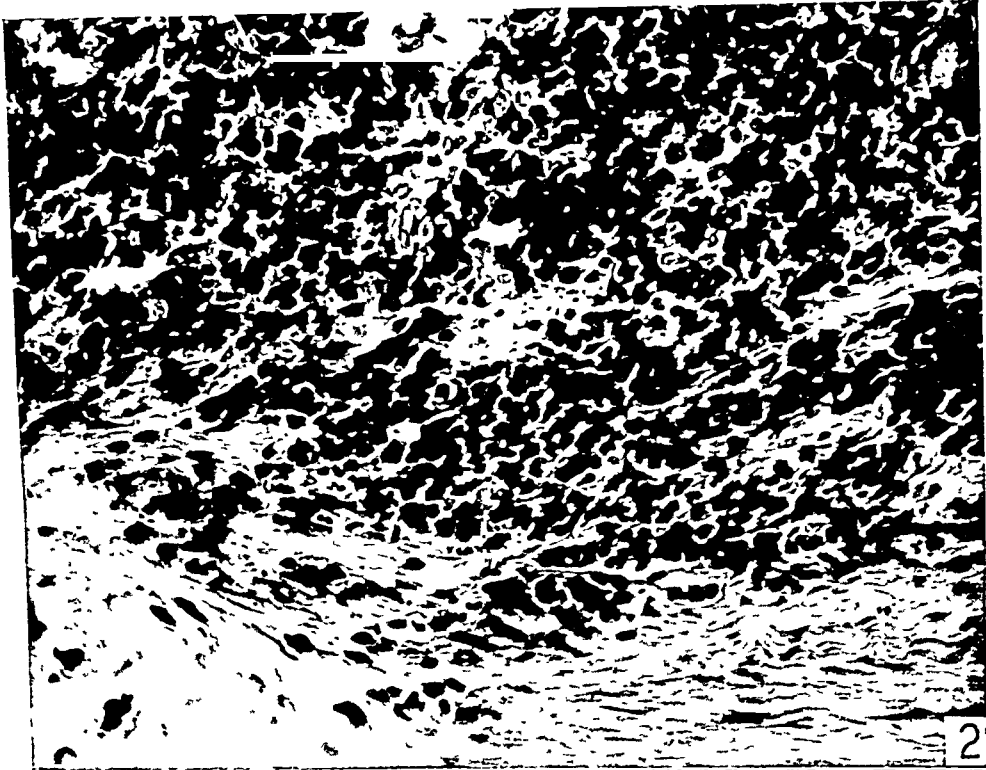




## PLATE 7

FIG. 2. The edge of the invading tumor of the previous section. The mast cells are so filled with metachromatic granules, appearing black in the photograph, that no detail in their cellular structure can be seen. This tumor contained fifty times as much heparin as normal dog liver. Dominici stain.  $\times 300$ .

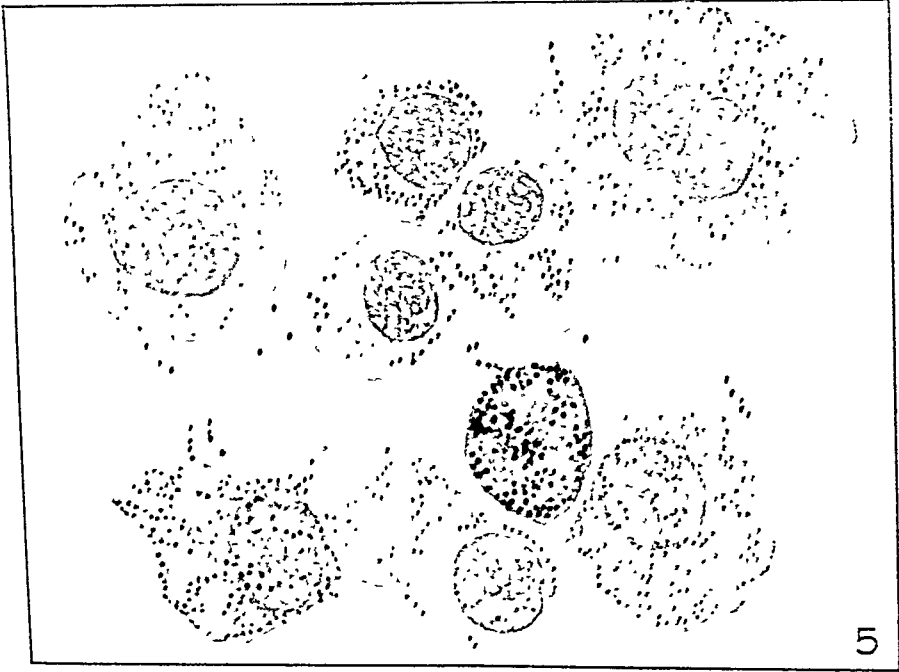
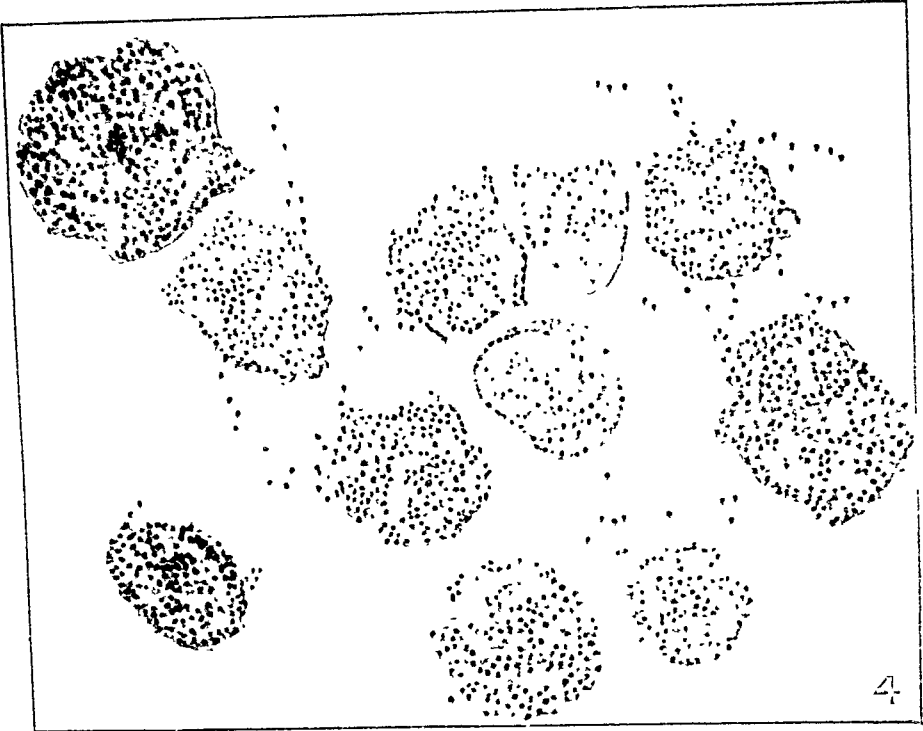
FIG. 3. Detail of the tumor of immature cell type from which the second preparation of heparin was made. Scattered through the section, particularly along its left and lower margin, is seen a sprinkling of black-stained mature mast cells similar to those of the preceding specimen. In the right half of the figure the tumor cells contain fine granulations which are invisible in the photograph so that they lack the appearance of mast cells. As is shown in Fig. 5, an imprint from this same tumor, these apparently clear cells contained a fine dust-like granulation. The growth yielded about one-thirtieth as much heparin as the mature tumor of Fig. 1. Dominici stain.  $\times 300$ .



## PLATE 8

FIG. 4. Imprint preparation from the mature mast cell tumor of Figs. 1 and 2, stained by Wright's method. The cells are filled to a greater or less extent with large metachromatic granules which in most cases obscure the light blue-stained nucleus.  $\times 1800$ .

FIG. 5. Similar preparation of the immature mast cell tumor. The anaplastic cells of the immature tumor have an ample vacuolated cytoplasm which contains many fine dust-like granulations. The nuclei are large and deeply metachromatic as compared with the light blue nuclei of the more mature cells in Fig. 4. In one cell nuclear division has occurred. In the lower center is seen one of the occasional mature mast cells scattered sparsely throughout the immature tumor.  $\times 1800$ .



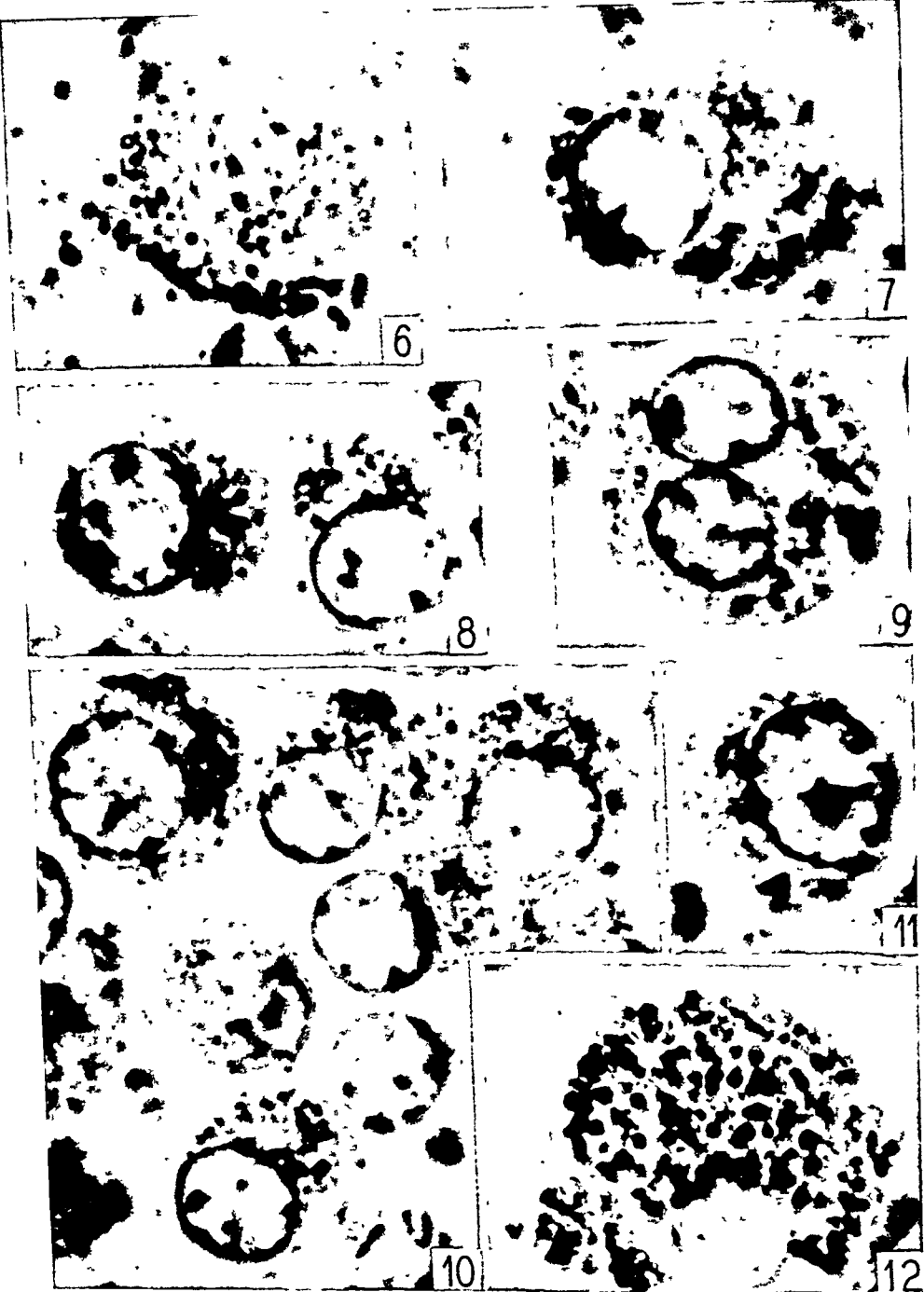
## PLATE 9

FIGS. 6 to 12. Photographs of living unstained tumor mast cells taken by phase contrast microscopy. Under such circumstances the diaphragm is fully open and the maximum contrast occurs only at the exact focus. Objects out of focus appear with diffraction rings (*vide* floating extracellular granules in Fig. 12). This means that the variations in the density of the intracellular particulate material are not the result of the common optical artifacts produced by cut-down illumination and out of focus examination, but that the considerable differences in appearance are optically real.  $\times 2860$ .

FIG. 6. Living unstained cell of the immature type. The cell has been slightly flattened by pressure. The cytoplasm is filled with particulate material presenting three optical appearances; densely black discrete granules, similar black granules with optically clear centers, and diffusely scattered greyish material in granular form. This last is in the same optical plane of focus as the sharply defined black granules.

FIGS. 7 to 11. Living mast tumor cells from the same specimen. In these preparations the cells were floating in fluid, so the cytological detail is less clear because of their relatively great thickness. All show diffusely distributed greyish particulate matter among which the dense black granules are scattered in irregular clumps.

FIG. 12. A living mast cell from a mature tumor. The cytoplasm is crowded almost to bursting with dense black granules and there is very little greyish particulate matter.







# THE MORPHOLOGY AND BEHAVIOR OF NEOPLASTIC MAST CELLS CULTIVATED IN VITRO\*

By GEORGE H. PAFF, Ph.D., FRANK BLOOM, D.V.M., AND  
CHRISTOPHER REILLY†

(From the Departments of Anatomy and Pathology of the  
Long Island College of Medicine, Brooklyn)

PLATES 10 AND 11

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Tumors composed of mast cells (1) have been shown to contain heparin in amounts that vary with the granular content of the neoplastic cells. This variation in the granules finds expression in the size, number, and tinctorial and optical properties of the cytoplasmic particulate matter (2). Cytological characteristics of this sort can be observed in great detail in tissue culture and furthermore the evolution and differentiation of the cell structures can be followed more advantageously under such conditions than in the original living tissues. Since the literature contains no comprehensive data relative to the morphology and behavior of mast cells in tissue cultures, this technique was therefore applied to two anaplastic mast cell tumors.

## Technique

The tumor tissue fragments were obtained from two dogs. In every instance one-half of the fragment was fixed in Zenker formol or 10 per cent neutral formalin and sectioned and stained in hematoxylin and eosin, iron-hematoxylin, Mallory's aniline blue, and other stains; the other half was placed in several cubic centimeters of whole dog's blood under aseptic conditions for later cultivation *in vitro*. This latter procedure was followed because the tissue had to be transported across the city from the operating room to the tissue culture laboratory. Once there, the fragments were washed free of blood in Tyrode solution and then placed in dog serum for subdivision preparatory to planting in hanging drops and roller tubes.

The hanging drop cultures were prepared by mixing one drop of chicken blood plasma, two drops of normal dog serum, and one drop of chick embryo extract. Before clotting occurred, a fragment of tumor was added. In making roller tube cultures, the tubes were first lined with chicken blood plasma, ten to fifteen small tumor fragments were then set in the lining, and two drops of embryonic extract added. After clotting occurred, each tube received 2.5 cc. of normal dog serum and 0.5 cc. of chick embryo extract. All cultures were incubated at 38°C. and the drum which supported the roller tubes revolved ten times per hour. With both the hanging drop and roller tube cultures transplantation was done as seemed necessary because of beginning plasma liquefaction or to prevent deterioration of the cells.

Toluidine blue, a basic aniline dye, was used for staining the cells. This was done because "the only criterion by which a mast cell can be recognized is the presence in its protoplasm of certain granules which stain electively and metachromatically with basic aniline dyes" (3).

\* Supported by grants from the American Cancer Society.

† With the technical assistance of Miss Josephine Kristan.

The stain was prepared by adding to Tyrode solution enough grains of powdered toluidine blue to impart a sky-blue color to the solution. Hanging drop cultures were stained by freeing the paraffin-sealed coverslip and flooding the slip with the Tyrode-toluidine blue solution. No fixation was used since the prime object was to stain the granules metachromatically in the living cells. In the case of roller tubes the cultures were stained by adding the saline stain directly to the fluid medium or by replacing the fluid medium with the saline-toluidine blue solution. Either procedure gave a good result.

Some of the cultures were fixed in Zenker formol or 80 per cent alcohol and stained with Heidenhain's iron-hematoxylin.

In addition to direct microscopic examination of unstained and stained material, cinematographic continuous time-lapse photographs were made of individual cells as well as of cells in groups over periods varying from 6 to 12 hours.

### OBSERVATIONS

Tissue culture of the two tumors utilized revealed differences in growth, morphology, and behavior of the neoplastic mast cells, and hence each growth will be described separately.

*Tumor I.*—A 9 year old male Boston terrier was brought to the hospital for surgical removal of a cutaneous nodule lying over the crest of the left ileum. This growth had been present for a year and after removal was identified as a mast cell tumor. Three weeks later the dog was returned to the hospital with twelve new skin nodules distributed over the trunk. No further surgery was attempted except to obtain biopsies for tissue culture purposes. The animal remained under observation for 4 weeks and became so cachectic that the owner requested its destruction. During this time the number of growths had increased to forty-five. These were subepidermal spherical nodules scattered over the surface of the trunk but not on the legs. In size the growths varied from 4 to 18 mm. in diameter and were elevated 2 to 4 mm. above the surrounding normal skin. The superficial epithelium was usually ulcerated and the nodules appeared greyish-tan on section. The left axilla was filled with an ulcerated mass measuring 14.5 cm. in diameter and 7.0 cm. in thickness. This large growth had developed within a period of 7 weeks. Autopsy showed small metastatic nodules up to 1.0 cm. in diameter in the liver, spleen, and lungs.

The tumor nodules consisted of dense collections of closely packed mast cells which had invaded the corium and subcutaneous tissues. Occasional neutrophils, eosinophils, lymphocytes, histiocytes, and plasma cells were scattered among the neoplastic cells. A capsule and trabeculae were absent and there was a sparse fibrous stroma.

The neoplastic mast cells were round, oval, or polyhedral and the round or oval nucleus was either centrally or eccentrically located in the cytoplasm. The nuclear structure was somewhat vesicular and one to three acidophilic nucleoli were present. There was considerable variation in cellular dimensions and the nuclei of the larger cells were usually hyperchromatic, with large nucleoli and often a bizarre shape. Nuclear hyperchromatism and hypertrophic nucleoli occurred not uncommonly in smaller cells as well. Cells of great size containing from two to eight nuclei were not unusual and there were many abnormal mitotic figures. Sections stained with toluidine blue revealed a relatively few reddish-purple, fine, delicate metachromatic granules. In the

occasional mature mast cell present in the same section, the granules were coarser and usually so numerous as to obscure the nucleus.

Imprints of the tumor treated with Wright's stain more clearly demonstrated the granules and the quantitative variations that existed in the different cells. The granules had a fine delicate appearance that contrasted with the coarser granules seen in imprints of normal mast cells.

*Cultivation in Vitro.*—Tumor I grew very slowly in tissue culture and the lag phase was greatly prolonged; no growth was observed in hanging drop or roller tube culture until 8 to 10 days or more had elapsed. During this phase no cells of any kind wandered out into the medium. When migration and growth were finally discernible the type of growth in the tubes differed from that in hanging drops.

In the roller tubes the first indication of growth activity was the outward advance of a few contiguous cells at one or two points along the circumference of the fragment. The growth had an appearance not unlike epithelium, the cells extending out in a single layered sheet. The shape of the cells varied, but on the whole they were polygonal or rounded. No long spike-like protoplasmic processes were present (Fig. 1).

The first cells to grow out contained only one nucleus, but with extension of the sheet of tissue some with two and three nuclei appeared. Fig. 1 shows such a sheet as it appeared after 16 days' incubation of a roller tube. The cell shapes and number of nuclei are faithfully represented but the low magnification did not permit accurate representation of cytoplasmic structures.

The most striking feature of the cells was the appearance of the cytoplasmic granules; even in the unstained condition their uniform size and great number were evident. When stained with toluidine blue they assumed the violet color of metachromasia and contrasted sharply with the faint blue of the nuclei. Every cell of the growth showed these same distinctive cytoplasmic structures. Only mast cells had grown out so that these sheets of tissue represented pure cultures, despite the fact that the original tumor fragment contained connective tissue and other cellular elements removed from the skin and tela subcutanea.

Hanging drop cultures made from the same material presented a quite different picture. The cells extended out in strands and a great range of shapes was encountered, varying from the spindle form to elements with many processes (Figs. 2 to 4). Some of these last were eight to ten times as long as the cell body and exceedingly thin; when cytoplasmic granules were present in them these lay in single file. Other processes were more dendritic in appearance, tapering rapidly from a broad base to a point from which a thin extension, often of considerable length, arose (e.g. Fig. 5).

The cells in the hanging drop cultures held granules varying considerably in size, though in any given cell all had nearly uniform dimensions. Only oc-

casionally one could perceive within a single cell granules which varied from barely visible to coarse (Fig. 6). When stained with toluidine blue the granules gave the violet color characteristic of metachromasia. With the technique used, not all of them were stained at the same time, those in the peripheral cells of the colony usually staining first. To stain those close to the original fragment exposure to the dye for several hours was sometimes necessary and by this time the color of the cells which had stained first had faded. Sometimes not all of the granules in a single cell could be stained. Some of the granules in one part of a cell would remain free of dye, while granules of similar appearance in adjacent areas of the same cell were deep violet (Fig. 3). In a few elements none of the granules could be stained despite the fact that morphologically they were identical with adjacent cells which showed all granules metachromatically stained.

Within many of the proliferating mast cells an area free from granules was present adjacent to the nucleus (Fig. 4). In the unstained cell close examination was necessary to distinguish this area from the nucleus and only in stained preparations was it quite clearly cytoplasmic.

Cinematographic studies were made on the hanging drop preparations. By time-lapse exposure technique it was possible to demonstrate unmistakable ameboid movement. Some mast cells which had grown out from the original fragment could be seen to return toward the fragment or to move among stationary neighboring cells whereas other cells failed to retract their protoplasmic processes or change shape appreciably over an observation period of 6 hours. Division was seen to occur only by amitosis; no mitotic figures were observed. Preliminary to division it was noted that the protoplasmic processes were retracted, the cell became rounded, and the granules more refractile, with result that the cytoplasm transmitted light less freely. Using these criteria one could predict division. Amitosis followed with the formation of two daughter cells of equal size, which soon moved away from each other and again extended their protoplasmic processes into the surrounding medium.

*Tumor II.*—Since this is the same tumor described in the preceding paper (2), a mastocytoma of immature cell type, the history and gross description of the tumor need not be repeated. Microscopically it consisted of dense collections of mast cells which were scattered below the pars papillaris of the corium and infiltrated the subcutaneous tissues. There was an irregular fibrous connective tissue stroma which in some regions showed trabecular formations. Some areas were heavily fibrosed and in others the connective tissue proliferation was minimal. Haphazardly distributed among the neoplastic cells were occasional histiocytes, neutrophils, plasma cells, eosinophils, and lymphocytes.

In general the tumor cells were similar to those of Tumor I. Cellular pleomorphism was as prominent but mitotic figures were fewer and of the normal type. Staining with toluidine blue indicated that the metachromatic granules were somewhat more plentiful and that they resisted decolorization more strongly. The granules had the same fine delicate structure as those of

the preceding tumor and hence contrasted with the coarser granules of normal mast cells and of the tumor mast cells first reported by Bloom (1).

In imprint preparations, the granules were essentially similar in number and structure to those in Tumor I.

*Cultivation in Vitro.*—2 days after planting pieces of Tumor II in roller tubes an excellent outgrowth of cells appeared from the entire circumference of some of the original fragments. The cells did not grow out as contiguous elements having the appearance of an epithelium but as spindle-shaped cells. Under low magnification the cultures therefore looked much like cultures of fragments from the ventricle of the 8 day chick embryo heart. Under higher power, however, the spindle-shaped cells were seen to contain the characteristic cytoplasmic granules which stained purple with toluidine blue. The granules were especially numerous in the cell body surrounding the nucleus, though occasionally a granule-free area lay adjacent to the latter.

The hanging drop cultures also showed differences between the growing cells of this tumor and those of Tumor I. As in the roller tube, the lag phase was far shorter and the growth more rapid. Proteolytic activity was considerably greater as evidenced by the fact that the fibrin clot was more rapidly liquefied, an occurrence necessitating more frequent transplantation of the growing tissue to a fresh clot. Occasional mitotic figures were observed, a fact confirmed in sections stained with iron-hematoxylin. Finally, in Tumor II more cells were present which showed a mixture of granules ranging in size from bare visibility to coarse (Fig. 6). Cells filled with coarse granules alone were less numerous than in cultures from Tumor I.

#### DISCUSSION

From the standpoint of the phenomena of tissue growth in culture, the most peculiar and perhaps significant observation made in our cultures of the mast cell tumor is the fact that only mast cells grew. Several previous observations and conclusions lead to an interesting line of thought in this regard. Heparin has been shown by Fischer (4, 5), Goerner (6), and Zakrzewski (7-10) to inhibit cell growth. Jorpes (11) after reviewing the work of a series of investigators, has concluded that the granular substance of the normal tissue mast cells is heparin. A paper published herewith (2) has shown that a mast cell tumor may have as much as fifty times the heparin content of normal tissues, and has provided reason to suppose that the heparin content varies with the number and size of the metachromatic granules within the cells. If the cultivated cells were producing heparin *in vitro* it seems possible that this material acted to prevent growth of the other cells in the tumor fragment.

The metachromatic cytoplasmic granules are the most characteristic structures found within proliferating mast cells. Since their granules may be concerned in the elaboration of heparin, the steps involved in their formation and dissolution become important. Our observations in cultured cells closely

accord with those of Oliver, Bloom, and Mangieri as reported in the associated paper on the heparin content and the cytoplasmic particles of tumor mast cells (2). These investigators correlated the heparin content of the tumors examined with the occurrence in the cells of granules differing in size and number and with differing tinctorial and optical properties. Their finding suggests an elaboration of pre-heparin, finely particulate matter into the coarse metachromatic granules of the mature heparin-containing cells. As yet we have not observed a complete cycle of granule formation within a single living cell, but the composite picture seen in the developing cells of the tissue culture lends itself to such an interpretation. Following staining with toluidine blue we were unable to detect within the nucleus either metachromatic granules (12) or a diffuse metachromasia (2, 12), findings which were the basis of Downey's conclusion that the granules are derived from the nucleus. In the cytoplasm, however, a sequence in granule formation is strongly suggested by the presence of fine and coarse granules and a corresponding variation in tinctorial reaction from a complete absence of metachromasia in the fine granules to the development of its intensely reddish-purple color in the coarse ones. The optical differences in the granules demonstrated by phase microscopy, noted in the living cells of the tumors (2), were also seen in the tissue cultures (Fig. 7).

Many observers believe that division in normal mast cells is solely by amitosis (13, 14). This was true in the cultures of one of the tumors we have studied, but division by mitosis was observed as well in the culture of the second tumors, an occurrence which may have been due to the relatively more anaplastic state of the neoplastic cells.

#### SUMMARY AND CONCLUSIONS

Fragments from two mast cell tumors of the dog have been cultured *in vitro*. Studies on the living and on fixed and stained preparations revealed the following:

Only mast cells grew out from the original tumor fragments though these contained other types of cells. They grew in some of the roller tube cultures in a sheet resembling an epithelium but in hanging drop cultures they lay separate and were irregularly spindle or star-shaped with long protoplasmic processes.

The cytoplasmic granules of the proliferating mast cells varied in size, number, and tinctorial properties. In most of the cells they stained metachromatically, in occasional cells some of the granules only could be stained, and in a few none could be stained.

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## EXPLANATION OF PLATES

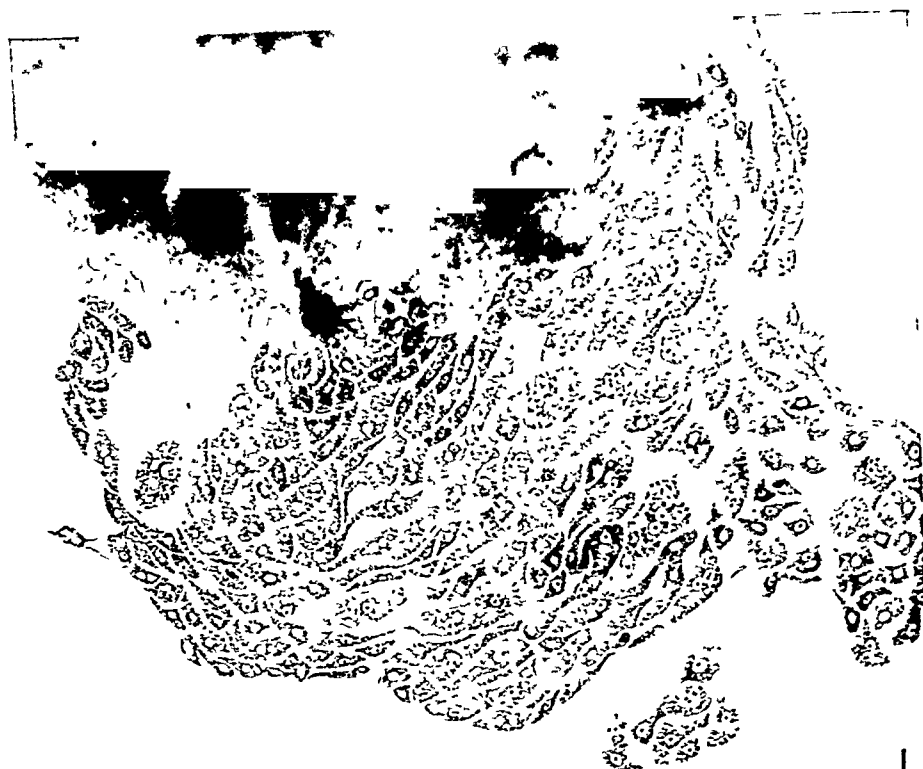
## PLATE 10

FIG. 1. Tumor I. Sheet of mast cells resembling epithelium. Stained with toluidine blue after 16 days' cultivation in roller tube. Drawing,  $\times 100$ .

FIG. 2. Tumor I. Group of mast cells cultured for 63 days in a hanging drop. In some of the cells the metachromatic granules are especially numerous about the nucleus. Toluidine blue.  $\times 100$ .

FIG. 3. Tumor I. Three mast cells from a fragment grown for 55 days in a hanging drop culture. All of the granules in the central cell have taken the stain deeply whereas those of the cell on the left differ widely in this respect. Toluidine blue.  $\times 430$ .

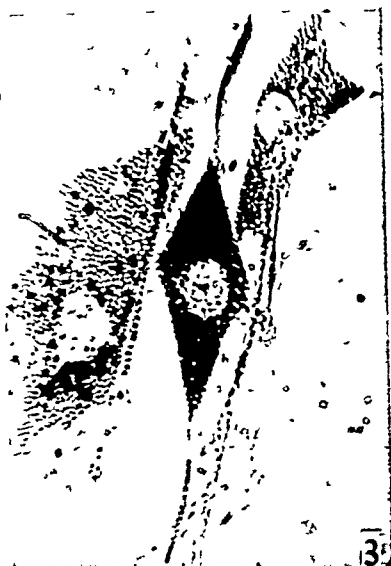




1



2



3

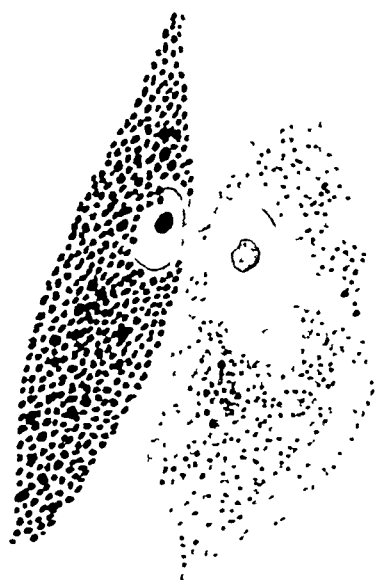
## PLATE 11

FIG. 4. Tumor I. Mast cells grown 66 days in a hanging drop culture. Note cell processes, distribution of metachromatic granules, and the cytoplasmic area free from them near the nucleus. Toluidine blue.  $\times 200$ .

FIG. 5. Tumor II. Mast cell from a hanging drop culture 45 days old. Note the size and distribution of granules and the long tenuous processes. Toluidine blue.  $\times 430$ .

FIG. 6. Tumor II. Two cells from a 41 day old culture stained with Heidenhain's iron-hematoxylin. Note coarse granules in cell at left and fine granules in cell at right. Drawing,  $\times 970$ .

FIG. 7. Tumor III. A photograph taken with the phase microscope of two living spindle-shaped mast cells containing coarse granules, from a tissue culture. Some of the granules appear homogeneous, whereas others have a shell of dense material surrounding a clearer central portion. Hanging drop, 11 days old.  $\times 970$ .





# AMINO ACID COMPOSITION OF HIGHLY PURIFIED VIRAL PARTICLES OF INFLUENZA A AND B\*

By C. A. KNIGHT, Ph.D.

*(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey)*

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The mutation of viruses to form new strains (1, 2) is a phenomenon which has been recognized for many years in the form of its various biological manifestations, but only recently has it become possible to attempt a correlation of this knowledge with the fundamental chemistry of the viruses themselves. Thus, amino acid analyses made on strains of tobacco mosaic virus have revealed differences in the composition of the virus proteins which presumably can explain their different biological properties and which also conceivably illustrate the nature of the chemical changes which accompany the mutation of a virus to form a new strain (3-7). In view of these findings, it was naturally of interest to determine whether or not similar chemical differences exist between strains or types of an animal virus. Highly purified preparations of influenza viruses (8-12) were available for this purpose and the PR8 strain of influenza A and the Lee strain of influenza B were chosen for comparison. These viruses produce clinically indistinguishable diseases (13) and appear to be very similar in gross chemical properties (9). However, they are serologically and immunologically distinct (13) and seem to differ slightly in size (12, 14). In this communication there are presented the results of an attempt to discover, at least in part, a chemical basis for the similarities and differences between these two types of influenza viruses. The approach employed has centered upon the protein components of the viruses and microbiological assays for amino acids have been made on hydrolysates of the highly purified viral particles of PR8 and of Lee influenza viruses obtained from the allantoic fluids of infected chick embryos.

## *Methods and Findings*

*Preparation of Virus for Assay.*—Highly purified preparations of the PR8 and Lee strains of influenza virus were obtained from allantoic fluids of infected chick embryos by a combination of the methods of differential centrifugation and adsorption on and elution from chicken red cells (8-12). Such preparations were found to consist of particles which were highly active biologically and which were uniform in size, in electrochemical behavior, and in serological reactions (11). The purified viruses were freed of salt and dried as recently described (15). Hydrolysates of the viruses were obtained by heating 50 mg. samples in 2 ml. portions of 2.7 N hydrochloric acid in sealed tubes in an autoclave at 15 pounds pressure for 10 to 12 hours.

\* Presented in part at the meeting of The American Society for Biological Chemists in Chicago, May 18 to 22, 1947.

The hydrolysates were neutralized and filtered and the combined filtrate and washings for each sample was brought to a volume of 250 ml. For the tryptophane assays, separate samples of 11 to 15 mg. were hydrolyzed in 1 ml. portions of 20 per cent sodium hydroxide in sealed tubes in an autoclave at 15 pounds pressure for 15 hours. The hydrolysates were neutralized, filtered, and brought to a volume of 100 ml. For the sake of comparison, an hydrolysate of the sedimentable component of normal allantoic fluid (16) was also prepared and simultaneous assays were made on this and the virus hydrolysates.

TABLE I

*Amino Acid Content of Highly Purified PR8 and Lee Influenza Virus Particles and of the Sedimentable Particles of Normal Allantoic Fluid*

Amino acid	PR8 influenza virus	Lee influenza virus	Normal allantoic particles	MD*
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Alanine.....	2.5	2.6		0.1
Arginine.....	5.0	4.0	3.9	0.2
Aspartic acid.....	7.4	7.3	6.2	0.1
Glutamic acid.....	7.7	6.2	6.1	0.2
Glycine.....	2.5	2.9	1.8	0.1
Histidine.....	1.4	1.5	0.8	0.03
Isoleucine.....	5.2	5.4	4.1	0.1
Leucine.....	5.3	5.5	4.3	0.1
Lysine.....	3.6	4.7	2.5	0.1
Methionine.....	2.3	2.1	1.1	0.1
Phenylalanine.....	3.7	3.4	3.6	0.2
Proline.....	2.6	2.7	2.8	0.2
Serine.....	2.2	2.2	2.1	0.1
Threonine.....	3.7	4.0	3.8	0.1
Tryptophane.....	1.1	0.7	0.7	0.02
Tyrosine.....	3.1	2.1	2.2	0.05
Valine.....	3.4	3.2	3.2	0.1

\* Mean deviation of the values of single determinations from the averages given in the table.

*Microbiological Assays.*—The methods used were largely those of Stokes and co-workers (17-19), to whom the author is also indebted for original cultures of the bacteria employed. Alanine, glutamic acid, proline, and glycine were not determined by Stokes and collaborators. However, they were determined in the present investigation by microbiological assays which satisfied fairly well the usual criteria of reliability in this type of analysis (17). Thus, *Streptococcus faecalis* was employed to assay for alanine, arginine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, threonine, tryptophane, and valine. *Lactobacillus delbrückii* LD5 was used to determine phenylalanine, serine, and tyrosine; and *Leuconostoc mesenteroides* P-60 (20) was employed in assays for proline, aspartic acid, glycine, and in some instances, for lysine. The basal medium of Stokes (18) was used in all cases.

Five preparations of PR8, four preparations of Lee virus, and a combination sample representing several preparations of normal allantoic particles were analyzed. The averages of the results obtained in these analyses are given in Table I. From the mean deviations, also presented in Table I, one can obtain an estimate of the reproducibility of each analysis and

hence a judgment of which of the observed differences are probably significant. On this basis, the values shown in Table I indicate significant differences between the PR8 and Lee influenza particles in their contents of arginine, glutamic acid, lysine, tryptophane, and tyrosine. There may also be significant differences in the glycine and histidine values for the two strains, although the present data do not clearly indicate this.

#### DISCUSSION

In evaluating the differences observed in analyses made on the whole particles of influenza viruses, account must be taken of their chemical complexity. The highly purified particles of PR8 and Lee viruses contain protein, polysaccharide, lipid, and nucleic acid components. The sums of the percentages of these last three components are essentially the same for the two strains and the percentages of nitrogen contained in the intact virus particles, which can be determined more precisely, coincide (9, 21). From these facts, one can conclude that both strains probably contain identical quantities of protein and hence that the differences observed in amino acid assays of the whole particles are real and do not merely reflect variations in quantity of the non-protein constituents. This conclusion is strengthened by the nature of the analytical results. Five differences were observed and one of these was in the opposite direction from the other four. No significant differences were observed with respect to the value for ten of the seventeen amino acids determined. Therefore, the assumption that PR8 and Lee virus particles contain equal quantities of protein appears to fit the facts better than the alternative hypothesis. Furthermore, it should be noted that even if this assumption should prove false, the major premise of the report, namely that the protein components of the two strains of virus are markedly different in composition, still holds.

The analysis of highly purified preparations of influenza viruses has revealed, as in the case of similar analyses made on plant viruses, a noteworthy uniformity in the composition of successive preparations. The compositions of the PR8 and Lee influenza viruses were so characteristic of the strains used in these studies that they could undoubtedly have been used to identify them, as has been done with certain strains of tobacco mosaic virus (5). While the differences found in the present study are pronounced both in number and kind, it is interesting to note that the two strains appear to contain identical quantities of at least ten different amino acids. It seems that this should provide a chemical basis for the biological fact that these are influenza viruses, although more data would be required to establish this point firmly. In this connection, it can be seen from the results shown in Table I that the composition of the sedimentable particles of normal allantoic fluid closely resembles that of Lee virus in eight or nine cases and that of PR8 virus in four or five respects. However, a close and perhaps fundamental relationship has been established among these materials by immunochemical studies (11).

The present findings resemble those obtained in studies made on strains of a

plant virus, tobacco mosaic virus, both in character and in extent. In both instances the protein components have been found to differ. The results of the plant virus analyses demonstrated the presence of only a few differences between closely related and many deviations among presumably distantly related strains (3-7). The well known immunological distinction between the viruses of influenza A and B (13, 22-24) strongly suggests that they are not closely related, and it was found in the present analyses that the protein components of PR8 and of Lee viral particles differed in five or more rather than in one or two respects.

On the basis of current knowledge one can only speculate regarding the relationship of the present findings to the different biological and physicochemical properties of PR8 and Lee viruses. However, in the absence of data to the contrary, it seems reasonable to assume that a substantial portion of the serological activity and of the biological specificities of these viruses can be attributed to the protein components, which constitute approximately two-thirds of the weight of the viral particles. For example, the differences found herein might well account at least in part for the lack of immunological relationship between the two strains (13, 22-24), for their different pH stability ranges (25), their different red cell agglutinating capacities (11), and for the widely divergent heat stabilities of their agglutinating capacities (26). However, it is apparent that further studies of the sort reported herein will be required before it will be possible to draw more specific conclusions regarding the relationship of viral composition to viral properties.

#### SUMMARY

Microbiological assays for amino acids were made on hydrolysates of four to five highly purified preparations each of influenza A virus (PR8 strain) and influenza B virus (Lee strain). The results of the assays indicated that these strains of influenza virus contain approximately the same amounts of alanine, aspartic acid, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, and valine. However, significant differences were found in the values for arginine, glutamic acid, lysine, tryptophane, and tyrosine. It is believed that these differences may provide, at least in part, a chemical explanation for some of the differing properties of the PR8 and Lee strains of influenza viruses.

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# THE SUPERINFECTION OF THE RABBIT PAPILLOMA (SHOPE) BY EXTRANEOUS VIRUSES\*

By JEROME T. SYVERTON, M.D., AND GEORGE PACKER BERRY, M.D.

*(From the Department of Bacteriology, The University of Rochester School of  
Medicine and Dentistry, Rochester, New York)*

PLATES 12 TO 14

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A benign virus-induced tumor, the Shope rabbit papilloma (1), may undergo alterative changes in an expected and established sequence to become a malignant epidermoid carcinoma (2-6). The relation of the virus responsible for the initial growth to the final cancer, or to the cells of that cancer, is not known. At least four explanations for the relationship suggest themselves. Firstly, the papilloma and the ultimate cancer have the same etiologic agent, the papilloma virus; secondly, the papilloma virus becomes modified during the papilloma-to-carcinoma sequence and is present in the cells of the cancer in a changed or disguised form functioning as its essential cause; thirdly, the papilloma virus by initiating and actuating a growth with the immediate characters of a benign tumor, namely the papilloma, which ultimately undergoes carcinomatous degeneration, serves as a provocative carcinogenic agent; or, fourthly, the papilloma virus, once the growth becomes carcinomatous, is carried as a passenger in the cells of the cancer. The last possibility appeared to offer the best approach to a study of the rôle of the papilloma virus in the papilloma-to-carcinoma sequence. To explore one phase of this problem, we undertook to determine whether the cells of the virus-induced papilloma, or the cells of the cancers derived therefrom, could be superinfected by extraneous viruses; i.e., viruses that are extraneous in the sense that they have no etiologic relationship to the tumors under investigation. That cells integrant to the papilloma-to-carcinoma sequence adequately support the growth of extraneous viruses has been briefly recorded by Levaditi and Schoen (7) for the benign phase and by ourselves for both the benign and malignant phases (8). Moreover, multiple virus infection of individual host cells has been described (9, 10). The work on which our preliminary notes were based has been amplified by further experimentation and will be presented in detail in two papers.

The studies described in the present report were designed to establish the superinfection by extraneous viruses of the cells of virus-induced papilloma

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(cells that contain the Shope papilloma virus) and of an epidermoid carcinoma derived therefrom.

When we described our first studies (8), and when Levaditi and Schoen reported theirs (7), no evidence had been reported that cells containing a virus could be superinfected by unrelated viruses. Levaditi and Schoen described the superinfection of the epithelial cells of the virus papilloma (Shope) with vaccine virus, but they were unsuccessful when herpes virus was employed as the superinfecting agent.

In contrast to this limited use of virus tumors for infection by extraneous viruses, the thought of a virus being carried along in the cells of a cancer is not a new one. Levaditi and Nicolau (11) first reported that transplantable tumors of mice, rats, rabbits, and chickens supported the growth of viruses which had been introduced experimentally. Continuing these investigations, Levaditi and his associates (12, 13) successfully demonstrated the infection of a variety of tumors of unknown cause by many viruses (herpes, vaccinia, rabies, fowl pest, and lymphogranuloma venereum).

Further experimental work employing tumor cells for infection by viruses has been reported. Hallauer (14) found that tissue cultures of the cells of a mouse carcinoma would support fowl pest virus, whereas other mouse tissues would not. Mellanby (15) successfully infected tar- and dibenzanthracene-induced sarcomas of chickens with the Rous sarcoma agent by injecting the virus at a distant site. Although he was unable to demonstrate an alterative effect on the chemically-induced tumors, cell-free filtrates prepared from these tumors produced a Rous sarcoma. On the other hand, cells from these tumors on transplantation yielded tumors of homologous type from which virus was not recoverable. Findlay and MacCallum (16) found that yellow fever virus grew readily in the cells of a mouse carcinoma, and that certain strains of the virus could be carried for as many as 50 passages without any apparent change in their pathogenicity. The investigations of Rous and Kidd (17) have demonstrated the modifying influence of a virus on preexisting tumors of unknown cause. They describe remarkable effects as due to the infection of tar tumors of rabbits with the rabbit papilloma virus. The virus had an activating, transforming, and carcinogenic effect, whether the tar tumors were located superficially or followed implantation. Andrewes *et al.*, (18) reported single sarcomas originating in each of two rabbits after repeated intramuscular injections of tar and a single intravenous injection of fibroma virus (Shope). One of these sarcomas was transplanted serially, but repeated attempts to demonstrate fibroma virus either directly or by immunological tests were unsuccessful.

Histopathological evidence to suggest that a virus can be a superinfecting agent in tumors or even the responsible etiological agent has been presented occasionally. Thus, Russell (19) reported the presence of intranuclear inclusion bodies in 33 per cent of 192 human gliomata. Wolf and Orton (20) obtained similar results from a comparable study. Since the inclusion bodies that they described conformed in appearance to type B intranuclear inclusions, further work is needed to evaluate their findings.

Of significance to the present investigation is the well known discovery by Rivers and Pearce (21) that a transplantable malignant tumor of rabbits, the Brown-Pearce rabbit epithelioma, was spontaneously infected with a virus, virus III, and that this

virus was maintained and carried in successive tumor transplants even when the host was immune to reinfection with the same virus. Andrewes (22), moreover, found virus III occurring as a passenger in a transplantable sarcoma which originated at the site of intramuscular tar injections, and also as a superinfective agent in cells of the infectious fibroma of rabbits (Shope) (23). The extraneous virus, virus III, produced inclusion bodies in the fibroma cells, focal necroses of the tissues, and an accelerated regression of the growths. The presence of inclusion bodies in nearly all the cells of the passaged fibroma is natural evidence, supporting the experimental findings, that cells can support multiple infection by viruses (8-10). Andrewes observed further that complete suppression of the fibroma resulted when a mixture of fibroma virus and virus III was injected. This should not be interpreted as an "interference phenomenon," for the infection of the host cells by the virus of the shorter incubation period, virus III, would naturally destroy enough cells to make it difficult for the virus with the longer incubation period, fibroma virus, to propagate. This effect is similar to the suppressive or inhibitory effect which has been observed when a virus is injected at a site where the cells have been injured or destroyed as the result of exposure to Roentgen rays (24).

### *Materials and Methods*

*Viruses.*—Papilloma virus and five extraneous viruses each capable of producing readily recognizable and diagnostically significant inclusion bodies were employed.

The papilloma virus (Shope) (1) was obtained from papillomas that were present on cottontail rabbits (genus, *Sylvilagus*) when they arrived from Kansas, where they had become infected under natural conditions.

The Brazilian strain of infectious myxomatosis of rabbits (Sanarelli) (25) was employed. It was obtained from Dr. T. M. Rivers in whose laboratory at the Rockefeller Institute it had been carried in rabbits for a number of years. This highly virulent strain gives rise to a rapidly progressive disease which is characterized by rapid proliferation of cells with extensive metastases and death of the host. When examined histologically, the gelatinous tissue is found to consist of vascularized myxomatous tissue with large stellate cells. The epithelial cells in epidermis overlying the myxomatous tissue contain readily recognizable cytoplasmic inclusion bodies. Metastatic lesions in the skin of domestic rabbits were used to provide source virus.

The strain of B virus (26) had been obtained from Dr. A. B. Sabin. Its parenteral injection gives rise to a uniformly fatal encephalomyelitis in rabbits. Although the virus is predominantly necrotizing, with edema, hemorrhage, and infiltration by polymorphonuclear and mononuclear cells as secondary phenomena, type A intranuclear inclusion bodies can be seen in infected cells of the epithelium at the site of injection and in the central nervous system. We used tissue from the brain and cord of rabbits recently dead or moribund to provide source virus.

The virus III employed was a testicular-passage strain obtained from Dr. Rivers (27). This strain gives rise to local erythema, edema, tumefaction, a variable cellular infiltration, and type A intranuclear inclusion bodies in from 3 to 5 days after inoculation. Testicular tissue infected 4 days previously provided source virus.

The strain of vaccine virus employed was that used by the New York City Board of Health. It was maintained in our laboratory by testicular passage in rabbits and by passage on the chorio-allantoic membrane of chicks according to the Goodpasture technique (28). The virus, when used either in tissue suspension or as washed elementary bodies, produces typical

lesions with moderate necrotization and the formation of numerous cytoplasmic inclusion (Guarnieri) bodies in epithelial cells.

The highly virulent HF strain of herpes virus, isolated in 1922 by Flexner and Amoss (29), was employed. Although essentially neurotropic, this strain will attack cells derived from all three embryonic layers to produce microscopic changes which include the production of type A intranuclear inclusion bodies. The immediate source of virus for the present experiments was brain tissue of rabbits or mice that had died following inoculation of the virus by either the intracerebral or corneal routes.

*Animals.*—Eighty-three (*Oryctolagus*) and cottontail (*Sylvilagus*) rabbits were used in the present study. Six were normal rabbits; twenty-six had preexisting papillomas initiated 10 to 150 days previously by rubbing 0.1 ml. of virus suspension on a prepared, lightly scarified cutaneous site (approximately 2 cm. in diameter); one had an epidermoid carcinoma; and fifty were either immune or non-immune animals that were used to confirm the specificity of virus superinfections.

*Preparation of Virus Suspensions.*—The suspension to serve as inoculum was prepared from virus-containing tissues immediately after their removal. Using aseptic precautions, the tissues were weighed, ground in a mortar to a fine paste with an abrasive<sup>1</sup> and Locke's solution to yield the concentration desired. This suspension was centrifuged horizontally at 3000 R.P.M. for 30 minutes and the supernatant fluid, or a decimal dilution thereof, was used as the inoculum. The amount of inoculum and the route of injection are given in each protocol.

*Technique for Infecting Cells.*—Two methods were utilized in superinfecting papilloma cells with viruses. The method most frequently employed was that of infiltrative inoculation. Infiltration of a suspension through a small gauge needle (22 to 27 gauge) brought a single virus, or a mixture of two or three viruses, into immediate contact with susceptible cells and usually resulted in their parasitization.

A second method was employed to determine the tropism of papilloma cells for extraneous viruses. The virus was introduced at a site as far removed from preexisting papillomas as was conveniently possible and after a suitable interval attempts were made to elicit evidence of its presence in the papilloma cells.

*Fixation and Staining.*—Representative blocks of all infected tissues were secured for histopathological study immediately after the rabbits had been killed by inhalation of chloroform. The pieces of tissue were fixed in Zenker's (5 per cent acetic acid) fixative fluid and embedded in paraffin. The sections were stained with hematoxylin and eosin and according to Giemsa's method. Exceptionally, these stains were supplemented by phloxin-methylene blue and eosin-methylene blue.

#### EXPERIMENTS

That the cells of a virus-induced growth have been superinfected by one or more extraneous viruses becomes apparent only when these cells show intracellular changes characteristic of the extraneous agent. When careful histopathological study reveals such changes, one is justified in inferring that the extraneous virus is within that particular cell. Complementary evidence to show that a second virus is present, or even a third virus, are recovery of each virus by suitable animal passage and its identification by suitable immunological procedures. These criteria were employed in the present studies.

<sup>1</sup> Alundum, procured from Norton Company, Worcester, Massachusetts.

*Superinfection of the Cells of a Preexisting Virus Lesion by a Single Etiologically Distinct Virus*

Four preliminary experiments were planned to learn whether the epithelial cells of the benign virus-induced papilloma (Shope) can be superinfected by single extraneous viruses. The data and findings that relate to these four experiments (Experiments 1, 2, 3, and 4) are summarized in Table I.

TABLE I

*Results of Experiments 1, 2, 3, and 4*

*Proof that Cells of the Rabbit Papilloma Were Superinfected by a Single Extraneous Virus*

Experiment No.	Animal host			Superinfecting virus					Results					Inter- preta- tion
	Rabbit No.*	Papillomas		Name	Recip- rocal of dilu- tion	Amount	Duration of super- infection	Histologic examina- tion		Confirmatory tests			Proof of super- infection by extran- eous virus	
		No. infected	Age					Inclusion bodies		No. of rabbits em- ployed	Recov- ery of virus	Immu- no- logic tests		
								Intranuclear	Cytoplasmic					
			days		ml.	days								
1	DR1-1	1	37	B virus	10	1	6	+	-	2	+	-	Yes	
	DR1-2	"	"	"	"	"	"	0	-	"	+	-	No	
2	CR2-1	2	70	Vaccinia	100	"	5	-	+	6	+	+	Yes	
	DR2-2	"	"	"	"	"	"	-	+	"	+	+	"	
3	DR3-1	"	163	Virus III	10	"	6	0	-	2	+	+	?	
	DR3-2	"	"	"	"	"	"	0	-	"	+	+	?	
4	CR4-1	"	125	Herpes	"	"	10	0	-	"	0	-	No	
	CR4-2	"	"	"	"	"	"	0	-	"	0	-	"	

\* DR = domestic rabbit; CR = cottontail rabbit.

From the data summarized in Table I, it can be seen that the results of these first four experiments established the fact that epithelial cells of the virus-induced papilloma, cells that presumably contain papilloma virus, can be superinfected by a single extraneous virus (Figs. 1 to 4). Of the four viruses, B virus, vaccinia, virus III, and herpes virus, utilized for superinfection, B virus (Fig. 1) and vaccinia (Figs. 2 and 3) gave intracellular evidence of their presence. No specific evidence for superinfection by herpes virus or virus III resulted, on the other hand, when these viruses were used. Animal inoculation

and immunological tests verified the presence of virus III, but did not yield herpes virus in papillomatous tissue removed for examination 10 and 16 days, respectively, after such lesions had been infiltrated with herpes virus.

*Superinfection of the Cells of a Preexisting Virus Lesion by Two Distinct Viruses*

When it was learned from the first four experiments that a single virus can superinfect the epithelial cells of the rabbit papilloma, a second group of experiments (Experiments 5, 6, and 7) was planned to find out whether two extraneous viruses could simultaneously superinfect the papilloma, and, if possible, single papilloma cells. The data relating to these three experiments are presented in Table II.

From the findings of Experiments 5, 6, and 7, it was evident that the tumor readily supported the growth of two extraneous viruses, and that microscopic examination of single papilloma cells showed evidence for simultaneous parasitization by both viruses. A different combination of two viruses was employed for each experiment.

It can be seen from the results of Experiment 5 that the rabbit papilloma can be superinfected by B virus and myxoma virus injected simultaneously. Four of the five rabbits yielded evidence for the presence of intranuclear and cytoplasmic inclusion bodies in papilloma cells (Figs. 4 to 8). Moreover, in papillomas on two of these animals, both types of inclusion body appeared to be present in single cells (Figs. 5 and 6).

The results of Experiment 6 gave further evidence that papilloma cells can be readily superinfected by vaccinia virus. Evidence for infection by both vaccinia virus and virus III, on the other hand, was found in but a single animal (Fig. 9), and none of five animals yielded cells that contained both an intranuclear and a cytoplasmic inclusion body.

Experiment 7 yielded cytological evidence for the presence of vaccinia and B viruses in the superinfected papillomas (Fig. 10), but satisfactory evidence for the presence of both viruses in a single cell was not found. It was noted that the necrotizing effect of the two viruses in combination was great.

A further and final attempt was made in an eighth experiment to elicit intracellular evidence for the presence of two viruses in papilloma cells. This experiment was designed to determine what effects would result from the simultaneous infiltration of three viruses into preexisting papillomas on hosts that were immune to one of the viruses. It was hoped that an accelerated or hyperimmune generalized tissue reaction might advantageously affect the host's response to yield better results than those obtained previously. Accordingly, a mixture of myxoma, vaccinia, and B viruses was inoculated into papillomas on two rabbits that had recovered from infectious myxomatosis.



*Experiment 8. Superinfection with B Virus and Vaccine Virus in the Presence of an Immune or Accelerated Response to Virus.*—Sixty-four-day-old papillomas on two domestic rabbits

TABLE II

*Results of Experiments 5, 6, and 7*

*Proof that Cells of the Rabbit Papilloma Were Superinfected Simultaneously by Two Extraneous Viruses*

Experiment No.	Animal host			Superinfecting virus				Results					Interpretation		
	Rabbit No. *	Papillomas		Name	Reciprocal of dilution	Amount	Duration of superinfection	Histologic examination		Confirmatory tests			Proof of superinfection by		
		No. infected	Age					Inclusion bodies		No. of rabbits employed	Recovery of viruses	Immunologic tests			
								Intranuclear	Cytoplasmic						
5	DR5-1	4	days 147	B virus	See	1 ml.	5 days	+	+	-	-	-	Yes	Yes	Yes
	DR5-2	"	"	and	foot-	"	"	+	+	-	-	-	"	"	No
	DR5-3	"	50	myx-	note†	"	"	+	+	-	-	-	"	"	Yes
	DR5-4	"	20	oma	below	"	"	0	0	-	-	-	No	No	No
	DR5-5	"	"	virus		"	6	+	+	-	-	-	Yes	Yes	"
6	DR6-1	"	137		See	"	4	+	+	-	-	-	"	"	"
					foot-										
					note†										
					below										
	DR6-2	"	"	virus III	See	"	6	0	+	-	-	-	"	No	"
	DR6-3	"	50	and	foot-	"	3	0	+	-	-	-	"	"	"
	DR6-4	"	"	vac-	note‡	"	3	0	+	-	-	-	"	"	"
	CR6-5	"	"	cinia	below	"	17	0	0	4	+	+	?	?	"
7	DR7-1	"	25	B virus	See	"	5	0	0	6	+	+	Yes	Yes	"
	DR7-2	"	"	and vac-	foot-	"	6	0	0	"	+	+	"	"	"
				cinia	note†										
					below										

\* DR = domestic rabbit; CR = cottontail rabbit.

† Four papillomas on each rabbit were infiltrated with decimal dilutions,  $10^1$  through  $10^4$ , of a mixture consisting of equal parts of a 10 per cent suspension of B virus and 1 per cent suspension of the other virus employed.

‡ The injection of virus III was followed in 10 days by the injection of vaccinia virus.

that had recovered from infectious myxomatosis were infiltrated with a mixture made up from equal parts of 10 per cent tissue suspensions of three viruses, B virus, vaccinia, and myxoma virus. Both rabbits were acutely ill 48 hours later.

When rabbit DR8-1 was killed on the 4th day after injection, each lesion had an erythematous areola but no other change. Microscopic examination showed numerous, minute, circumscribed abscesses with many mononuclear and a few polymorphonuclear cells in the neighboring tissues, and a few Guarnieri bodies. No intranuclear or cytoplasmic inclusion bodies of the myxoma type were seen.

When rabbit DR8-2 was killed 5 days after its papillomas had been injected with the mixture of viruses, a marked inflammatory areola outlined the superinfected lesions and degenerative changes were apparent in the lesions that had received the 10<sup>1</sup> and 10<sup>2</sup> dilutions. Microscopic examination revealed marked changes in the subcutaneous tissues, where edema, dilatation of the blood vessels with some extravasation, polymorphonuclear infiltration, and areas of necrosis were present. The epithelial cells of the overlying epidermis were undergoing degenerative changes and a few contained typical type A intranuclear inclusion bodies such as are formed by B virus.

The greatly heightened tissue response of both hosts, and the demonstration of intranuclear inclusion bodies of type A in the papilloma cells of one rabbit and cytoplasmic inclusion bodies of vaccinia virus in the cells of the other rabbit, made it clear that the three viruses employed were active. In no instance, however, were inclusion bodies of both types present in a single cell, or even in sections taken from a single papilloma.

*Superinfection of the Cells of the Virus Papilloma of Rabbits with Virus  
III in an Effort to Establish Histological Evidence of Its Long  
Persistence*

Although we had been unsuccessful in demonstrating intracellular evidence for the parasitization of papilloma cells by virus III, its known affinity for the epithelial cells of the Brown-Pearce epithelioma (21) made it seem probable that a similar relationship could be shown for virus III and the cells of the virus papilloma. Accordingly, a ninth experiment was designed to find out whether virus III would parasitize the cells of the virus papilloma and thereafter maintain its association with them.

*Experiment 9. Superinfection of Papillomas with Virus III.*—Papillomas from 83 to 175 days old on each of five rabbits (DR9-1, DR9-2, DR9-3, DR9-4, and DR9-5) were infiltrated with virus III and biopsied for microscopic study 6, 8, 20, 84, and 120 days later. Microscopically, no evidence for the presence of virus III was observed.

The results of the ninth experiment gave no evidence that virus III had superinfected the epithelial cells of the virus papilloma when histological sections taken at intervals over a period of 4 months were studied.

*Tropism of Myxoma Virus for Epithelial Cells in Preexisting Papillomas*

After we had established that the direct introduction of viruses into papillomas on domestic and cottontail rabbits can result in intracellular evidence of superinfection of single papilloma cells, it became desirable to determine

whether the actively growing epithelial cells of these tumors would exert a tropic effect on an extraneous virus when it was introduced into the normal skin at a site distantly removed from the papilloma. Accordingly, a tenth experiment was undertaken, employing myxoma virus as the extraneous agent.

*Experiment 10. The Infection of Papilloma Cells by Introducing Myxoma Virus at a Site Removed from the Tumor.*—Four rabbits carrying actively growing papillomas were infected with myxoma virus, two by introducing intracutaneously 0.25 ml. of a  $10^4$  dilution of myxomatous tissue suspension at a site distantly removed from the papillomas, and the other two animals by placing them in a cage with a case of infectious myxomatosis.

Both rabbit DR10-1 with 231-day-old papillomas and rabbit DR10-2 with 10-day-old papillomas died of infectious myxomatosis 10 days after the virus had been injected intracutaneously. Portions of the test papillomas were removed for study. Microscopically, almost every cell of the basal layers had been superinfected by myxoma virus, as shown by the presence of cytoplasmic inclusion bodies characteristic of myxoma infections.

Rabbit DR10-3 had 30-day-old papillomas when it contracted rapidly fulminating infectious myxomatosis as result of accidental infection. Microscopically, most of the epithelial cells contained the typical cytoplasmic inclusion bodies of myxoma.

Rabbit DR10-4 carried 35-day-old papillomas when it contracted infectious myxomatosis by contact with its cage mate, DR10-3. Its general course and the microscopic findings were essentially the same as described for DR10-3.

The results of the tenth experiment constituted good evidence for the tropism of the epithelial cells of actively growing rabbit papillomas for myxoma virus. It was found that histological sections prepared from lesions on each rabbit showed numerous cytoplasmic inclusion bodies in almost all the epithelial cells of the basal layers.

#### *Virus Superinfection of the Cells of an Epidermoid Carcinoma Derived from a Virus Papilloma*

The successful demonstration of the ability of extraneous viruses to superinfect epithelial cells of the rabbit papilloma led us to determine whether cells representing a terminal phase of the papilloma-to-carcinoma sequence, cells of an epidermoid carcinoma, could be parasitized by an experimentally introduced extraneous virus, B virus, and, if so, to learn whether histopathological evidence of B virus infection would be present in the cells of metastatic lesions in lymph nodes and lungs. Accordingly, an eleventh experiment was carried out.

*Experiment 11. Superinfection with B Virus.*—Rabbit DR11-1, which carried an epidermoid carcinoma with metastases to the lymph nodes and lungs, was used. The primary carcinomatous lesions were infiltrated with a B virus suspension through a needle in the usual way. When the animal died of encephalomyelitis 7 days later, portions of the primary and metastatic carcinomatous lesions were removed for study. Microscopic examination confirmed the diagnosis of epidermoid carcinoma (Fig. 11), and revealed type A intranuclear inclusion bodies in the cancer cells (Figs. 12, 13, and 14). Moreover, many of these cells had undergone amitotic nuclear division from stimulation by B virus, which resulted in from 2 to

8 inclusion bodies within single cells (Fig. 14). Microscopic study of the metastatic lesions in lymph nodes and lungs, however, gave no evidence for infection by B virus.

The results of Experiment 11 made it clear that the epithelial cells of an epidermoid carcinoma (representing a final phase of the virus papilloma-to-carcinoma sequence) can be readily infected by an extraneous virus, B virus.

#### DISCUSSION

The experiments described herein were undertaken to learn whether a virus-induced tumor, the rabbit papilloma (Shope), and single cells thereof, could be superinfected by one or more extraneous viruses.

Our criteria for parasitization included the histopathological finding of specific inclusion bodies, the recovery of each virus by suitable animal passage, and the identification of each virus by suitable immunological procedures. Although these criteria could not be rigidly fulfilled in every case, we believe the evidence presented is adequate to establish the fact that papillomas, and single cells integrant to these tumors, were experimentally parasitized by one virus in some of the experiments and by two viruses in other experiments. A similar study of control tumors from the same animals, on the other hand, gave no evidence for the presence of an extraneous virus. Furthermore, since the cells of the rabbit papilloma presumably contain the specific virus responsible for this growth, our experiments seem to indicate that the cells of these growths can support coexistent parasitization by three different viruses.

Though the results of the present experiments strongly suggest, they do not prove that a single cell was parasitized by three viruses. Even though cytoplasmic and intranuclear inclusion bodies characteristic of the viruses used for superinfection seem to be in the same cell, it is possible in a stratified epithelial structure such as the rabbit papilloma that immediately adjacent cells overlying each other appear as a single cell—make it appear that two inclusion bodies, one cytoplasmic and the other intranuclear, are in a single cell whereas in reality each is contained within a different cell.<sup>2</sup>

Intracellular changes have been observed in papilloma cells which suggest natural superinfection of papillomas by viruses. Rous, Beard, and Kidd, (30) reported the presence of intranuclear inclusion bodies, both types A and B, in virus-induced rabbit papillomas which showed gross histopathological differences from the papillomas usually seen. Their attempts to transmit the agent

<sup>2</sup> In an attempt to obviate the possibility of erroneously concluding that multiple virus infection of a single cell had occurred because of observing inclusion bodies in cells overlying one another, we undertook further studies in which normal epithelial cells of the cornea were parasitized by several viruses. The results of this second investigation (9) satisfactorily substantiate the findings of the present investigation that single cells can be parasitized by several viruses at the same time.

supposedly responsible for the type B inclusion bodies were without success. We (8) too, have noted type B intranuclear inclusion bodies in the cells of certain papillomas; our efforts to evaluate their significance have been unsuccessful. As an explanation for our failure to demonstrate an extraneous virus, it occurred to us that the remarkable difficulty associated with the demonstration of papilloma virus in papillomas on domestic rabbits was not peculiar to the agent, but might also apply to extraneous viruses. It is possible, of course, that the type B inclusion bodies, which are so irregularly present, are non-specific in nature. This explanation seems most probable in view of the ease with which we have found that papillomas can be experimentally superinfected.

The present findings show that virus tumors, *per se*, are vulnerable to further attacks by extraneous viruses, obviously a matter of importance in the virus-tumor problem. It must be emphasized that the recovery of a virus from a tumor, or the presence in a tumor of histopathological changes secondary to virus activity, cannot be accepted as evidence of a specific etiological agent. Thus, we may consider virus tumors to be similar to non-virus tumors in their receptivity to parasitization by extraneous viruses. The extent to which this relationship can go is illustrated by the natural parasitization of the Brown-Pearce epithelioma by virus III (21).

It is clear that the cells of tumors can be easily infected under both natural and experimental conditions by viruses of no direct etiological significance. Moreover, these viruses can apparently be carried indefinitely by the tumor cells. It is obvious, therefore, that cognizance of this fact is essential to the investigation of any non-virus tumor, particularly since the presence of a virus in a tumor can mask the characteristic clinical and histological picture.

#### CONCLUSIONS

1. The potentialities that viruses have for the superinfection of virus tumors have not been recognized nor has the fact that a single cell can harbor more than one virus.
2. Rabbit papillomas, induced by the papilloma virus (Shope), were superinfected by B virus, myxoma virus, vaccinia virus, and probably, virus III. Similar attempts at superinfection by herpes virus were without success. The criteria for parasitization included the histopathological finding of specific inclusion bodies, the recovery of each virus by suitable animal passage, and the immunological identification of each virus.
3. Papillomas and probably the individual cells thereof were readily infected simultaneously by two viruses when the combination of B virus and myxoma virus was used.
4. Cells of the Shope papilloma have a selective affinity for certain extraneous

viruses introduced at a site distant from the tumor growths. It was found that exceptionally few cells in the basal layers of the epidermis escaped when myxoma virus was used as the superinfecting agent.

5. The cells of an epidermoid carcinoma which terminated the rabbit papilloma-to-carcinoma sequence were readily infected by B virus with resultant multiple intranuclear inclusion bodies in single cells.

6. Attempts to establish a prolonged superinfection of cells of the Shope papilloma by virus III were unsuccessful. This rabbit tumor differs therein from the Brown-Pearce tumor, an epithelioma of rabbits, in its susceptibility to infection with virus III.

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## EXPLANATION OF PLATES

The photographs were made by Mr. Merwyn C. Orser

## PLATE 12

*Superinfection of the Rabbit Papilloma by a Single Extraneous Virus (Table I)*

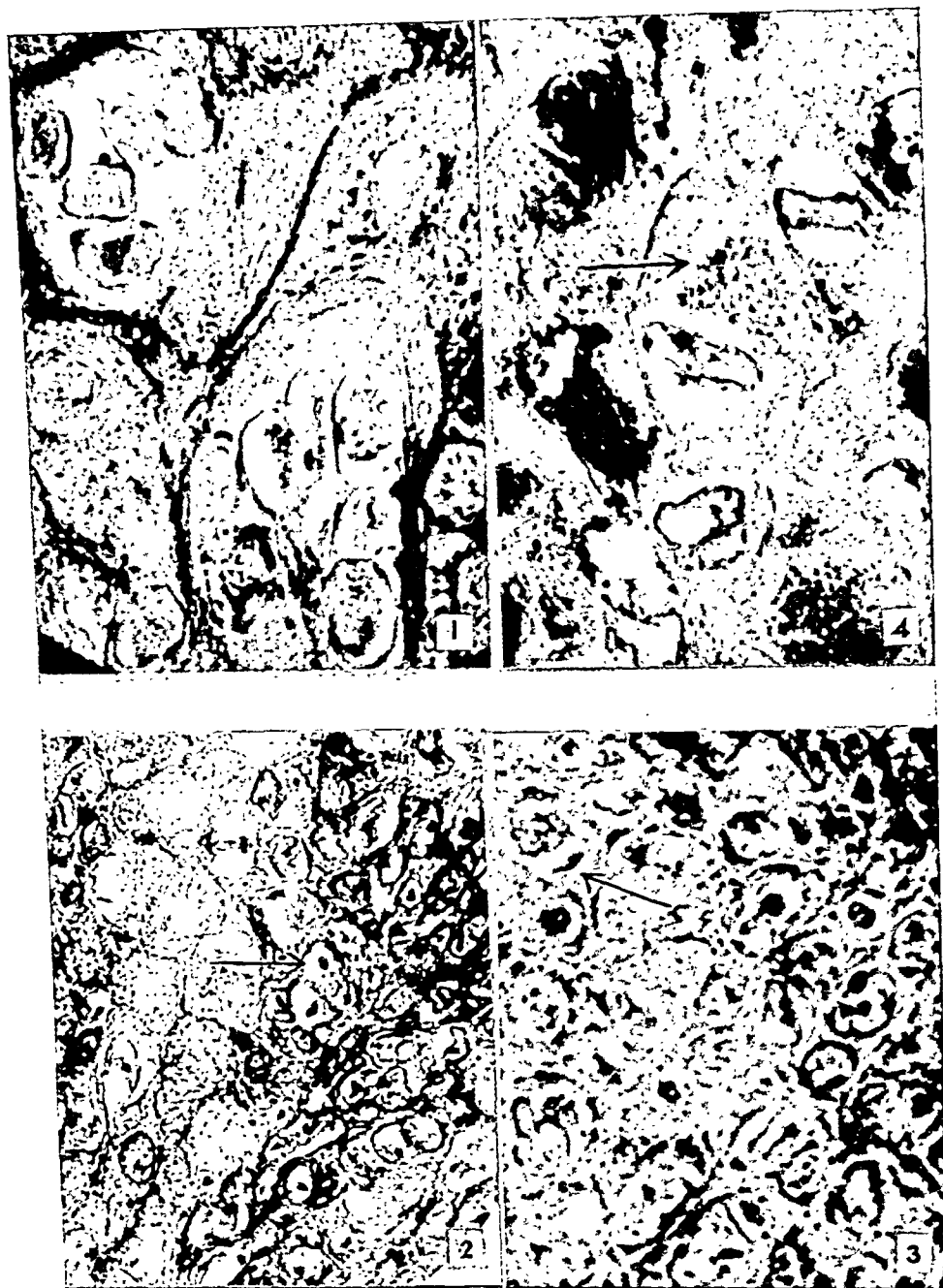
FIG. 1. Section of a papilloma from rabbit DR1-1 removed 6 days after infiltration with B virus suspension. Multiple type A intranuclear inclusion bodies are present in cells that have undergone amitotic nuclear division. Giemsa's stain.  $\times 1500$ .

FIG. 2. Section of a papilloma from rabbit CR2-1 removed 5 days after infiltration with vaccinia virus suspension. Cytoplasmic inclusion bodies (Guarnieri bodies) are present in most of the cells (arrow). Hematoxylin and eosin.  $\times 640$ .

FIG. 3. Section of a papilloma from rabbit DR2-2 removed 5 days after infiltration with vaccinia virus suspension. Many cells contain Guarnieri bodies (arrow). Giemsa's stain.  $\times 1500$ .

FIG. 4. Section of a papilloma from DR5-2 removed 5 days after infiltration with myxoma virus and B virus. Most of the cells show cytoplasmic inclusion bodies produced by the myxoma virus (arrow). Giemsa's stain.  $\times 1500$ .





(Syverton and Berry: Virus superinfection of papilloma cells)

## PLATE 13

### *Superinfection of the Rabbit Papilloma by Two Extraneous Viruses (Table II)*

FIGS. 5 and 6. Sections of papillomas removed from rabbits DR5-1 and DR5-3, respectively, 5 days after infiltration with a mixture of myxoma virus and B virus. Acidophilic intranuclear and cytoplasmic inclusion bodies are present in single cells (arrow). Giemsa's stain.  $\times 1460$ .

FIGS. 7 and 8. Sections of a papilloma removed from rabbit DR-5 six days after infiltration with a mixture of myxoma virus and B virus. Nearly every epithelial cell contains cytoplasmic inclusion bodies. Underlying the papilloma the myxomatous tissue (Fig. 7) shows many fibroblastic stellate cells containing type A intranuclear inclusion bodies. A single stellate cell with two intranuclear inclusion bodies is shown in Fig. 8. Giemsa's stain. Fig. 7,  $\times 210$ ; Fig. 8,  $\times 1460$ .

FIG. 9. Section of papilloma removed from rabbit DR6-1 four days after infiltration with a suspension of virus III and vaccinia virus. Acidophilic intranuclear inclusions and Guarnieri bodies in the cytoplasm are present in single cells (arrows). Giemsa's stain.  $\times 1460$ .

FIG. 10. Section of a papilloma removed from rabbit DR7-1 five days after infiltration with a mixture of B virus and vaccinia virus. Both intranuclear inclusion bodies and Guarnieri bodies are present in cells, but in no instance are both an intranuclear inclusion body and a cytoplasmic inclusion body surely present within a single cell. Giemsa's stain.  $\times 1460$ .

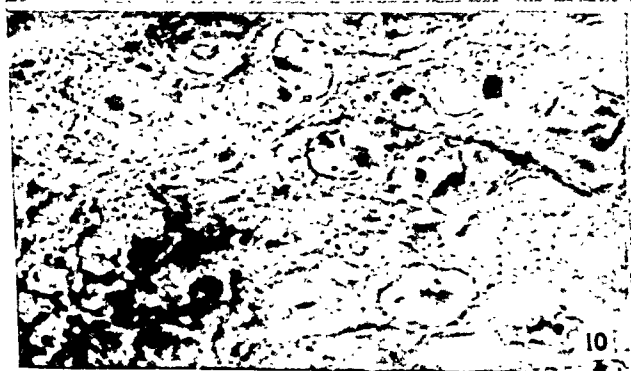
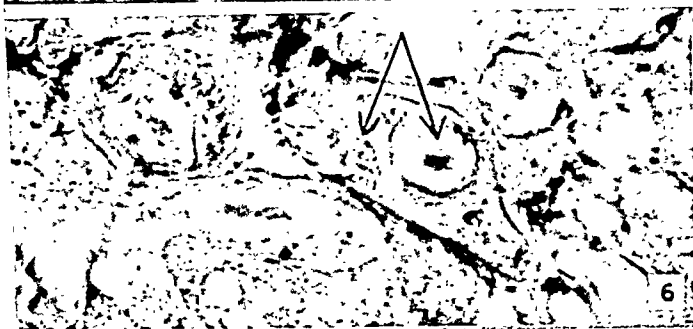
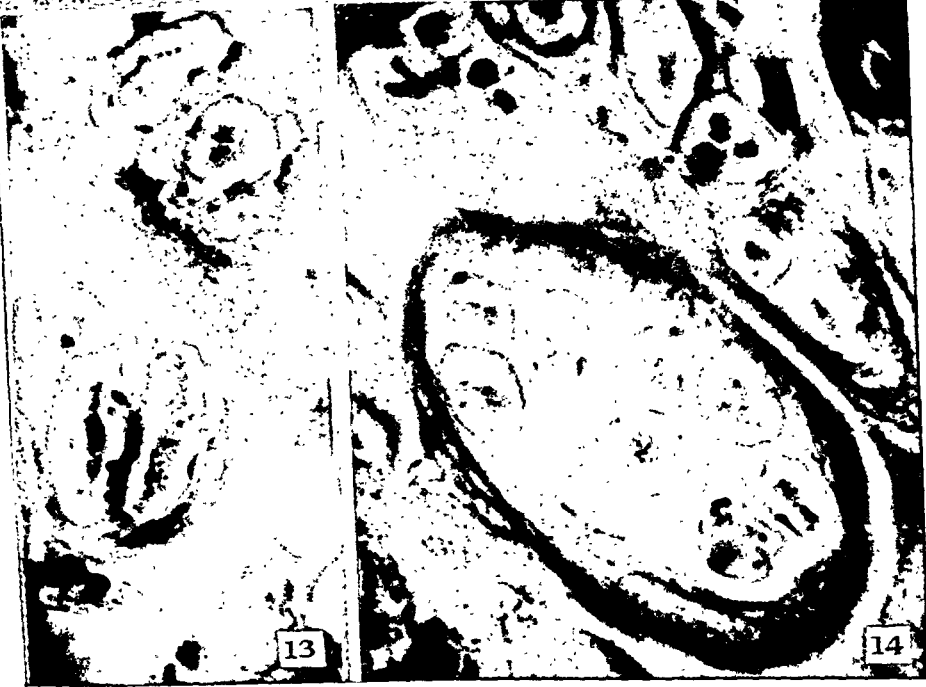
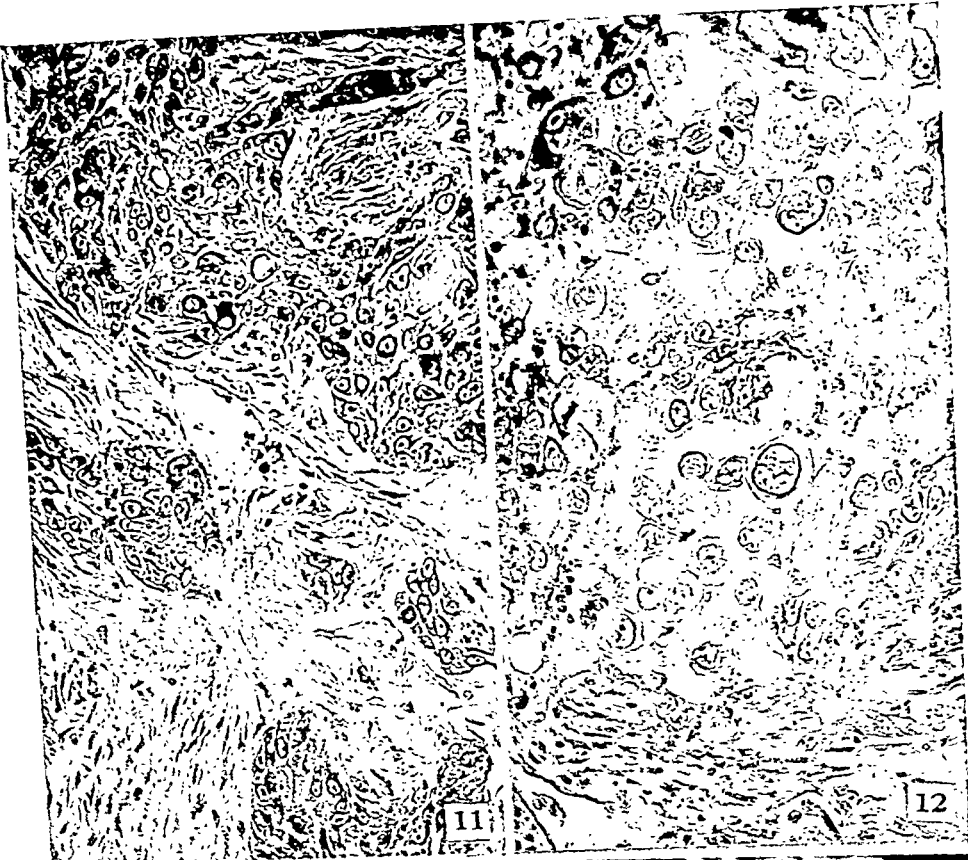


PLATE 14

*Superinfection by an Extranuclear Virus of an Epidermoid Carcinoma That  
Followed a Virus Papilloma*

FIGS. 11 and 12. Section of the primary carcinoma removed from rabbit DR11-1. Fig. 11 shows the cellular morphology of a portion of the cancer where there is no evidence of B virus infection and Fig. 12 shows hypertrophied cells most of which contain one or more intranuclear inclusion bodies. Hematoxylin and eosin.  $\times 210$ .

FIGS. 13 and 14. Cells from the section shown in Fig. 12 when viewed at a higher magnification. It can be seen that from one to eight intranuclear inclusion bodies are contained within a single nucleus. Giemsa's stain.  $\times 1500$ .



(Syverton and Berry: Virus superinfection of papilloma cells)



# MULTIPLE VIRUS INFECTION OF SINGLE HOST CELLS\*

By JEROME T. SYVERTON, M.D., AND GEORGE PACKER BERRY, M.D.

(From the Department of Bacteriology, The University of Rochester School of Medicine and Dentistry, Rochester, New York)

PLATES 15 AND 16

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In considering the intracellular parasitism that characterizes the host-virus relationship, it has often been tacitly assumed that the parasitization of an individual host cell is limited to infection by a single virus. In contradiction to this belief, however, we have reported experiments (1) in which the cells of a virus-induced tumor, Shope's rabbit papilloma (2), and of a carcinoma of the sort that frequently succeeds the benign growths (3, 4), were superinfected by other viruses—"superinfected" in the case of the papilloma because its cells contained the causative virus. These same studies, furthermore, included certain instances in which the simultaneous introduction of two viruses for the purpose of inducing superinfection was followed by evidence of the simultaneous activity of both of the viruses within individual cells. This evidence was the presence of two diagnostically significant inclusion bodies, the one cytoplasmic, the other intranuclear, within a single cell. In making our first report, we recognized that the histopathological examination of a stratified epithelial structure, such as the rabbit papilloma, involved the likelihood of error—of the mistaken interpretation that two overlying cells constituted a single cell. If such an error were made, the conclusion might be reached that the two inclusion bodies, cytoplasmic and intranuclear, were present in a single cell, when one of the inclusion bodies might actually be in one host cell and the other inclusion body in an overlying cell. It was for the purpose of eliminating, as far as possible, this opportunity for error that the present investigation was undertaken. The results of these experiments (5), which will now be described in detail, give evidence that an individual host cell can be simultaneously parasitized by more than one virus and amply confirm our earlier findings.

## Materials and Methods

The five viruses employed in the present investigations were vaccinia, myxoma virus, B virus, herpes virus, and virus III. The strains of virus employed, and the method for the preparation of the virus suspensions have already been described in detail (1 c).

Normal host cells for experimental infection were provided by using rabbit's cornea, skin, or testicle. Of the eleven rabbits employed, nine were domestic stock rabbits (*Oryctolagus*)

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\* This investigation was aided by a grant from The Jane Coffin Childs Memorial Fund for Medical Research.

and two were cottontails (*Sylvilagus*). The technique employed for the infection of corneal cells was to scarify both corneas by cross-hatching with a cataract knife, instill 0.2 ml. of virus mixture into the conjunctival sac of the right eye, and gently massage the cornea with the overlying lid. The scarified cornea of the left eye served as the control. The attempted infection of the cells of the skin and testicle was effected by infiltrative inoculation, as previously described (1 c).

Tissues for histopathological study were removed immediately after the rabbits had been killed by chloroform anesthesia. Representative blocks were fixed in Zenker's (5 per cent acetic acid) fixative fluid, and embedded in paraffin. The sections were stained with hematoxylin and eosin as routine, and according to Giemsa's method. Exceptionally, these stains were supplemented by phloxin-methylene blue and eosin-methylene blue.

#### EXPERIMENTAL

In an attempt to establish the infection of a single host cell by more than one virus, five experiments were undertaken. Each was based on the use of a different combination of viruses. The viruses in combination were selected so that one virus would give rise to inclusion bodies in the nucleus, and the other virus to inclusion bodies in the cytoplasm. Examples of such combinations are herpes virus and vaccine virus; B virus and myxoma virus.

#### *Simultaneous Infection of the Normal Cornea with Vaccinia and B Viruses*

Mixtures of vaccinia virus and B virus, after being prepared as indicated in the individual protocols, were used for Experiments 1 and 2.

*Experiment 1.*—Three domestic rabbits were used. The mixture of viruses utilized for instillation into the right conjunctival sac of each rabbit consisted of a 10 per cent suspension of virus-containing tissues. This mixture was prepared by combining 1 part of tissue containing vaccinia virus with 2 parts of tissue containing B virus. The vaccinia virus was derived from chorio-allantoic membranes of chicks. The B virus was obtained from the brain and cord of a moribund rabbit.

Two of the rabbits were killed 48 hours after inoculation. At that time keratitis was present in the right corneas of both rabbits. The left corneas showed negligible evidence of reaction along the lines of scarification.

*Rabbit A:* Examination of the sections revealed many Guarnieri bodies as evidence of vaccinia virus infection and many intranuclear inclusion bodies of type A as evidence of B virus infection. Both types of inclusion bodies were present in neighboring cells as well as in individual cells (Fig. 1).

*Rabbit B:* Although there were abundant inclusion bodies, both cytoplasmic and intranuclear, in different cells, in no instance could both a cytoplasmic and intranuclear inclusion body be demonstrated in the same cell.

*Rabbit C:* This rabbit was killed 72 hours after inoculation. Examination of the sections revealed extensive necrosis of the cornea and abundant evidence of specific virus activity in many of the remaining epithelial cells. Numerous cytoplasmic and intranuclear inclusion bodies were present. One area proved to be particularly worthy of careful study, for it revealed two cells separated from all the adjacent cells (Fig. 2). Within each of these cells a Guarnieri body and an intranuclear inclusion body of type A were found. Serial sections conclusively established the singleness of the cells in question and served further to rule out



the possibility of artefacts. These points were definitely established by photomicrographs taken in four optical planes in black and white (Fig. 3) and at three optical planes in color (Fig. 5).

*Experiment 2.*—One domestic rabbit, rabbit D, was used. The procedure followed that outlined above, except that the inoculum was composed of equal parts of a 10 per cent brain-cord suspension of B virus and vaccinia virus elementary bodies.

Study of the sections revealed many cells with either intranuclear (Fig. 6 a) or cytoplasmic (Fig. 6 b) inclusion bodies, but none in which both types of inclusion bodies were present.

From the findings described above, it is evident that the inoculation of two viruses among normal corneal cells is followed by both single and multiple virus infections of individual cells. Thus, following infection by a mixture of vaccinia and B viruses, multiple virus infections of single cells were discovered in two of four rabbits, while all four rabbits yielded evidence of single virus infections of cells by one or the other of the two viruses employed.

#### *Simultaneous Infection of the Normal Cornea with Vaccinia and Herpes Viruses*

In order to confirm and extend the findings which had been obtained in the first experiment, herpes virus was substituted for the more necrotizing B virus in Experiment 3.

*Experiment 3.*—Three domestic rabbits were used. The mixture of viruses consisted of equal parts of a 1 per cent testicular suspension containing vaccinia virus, and a 10 per cent brain suspension containing herpes virus.

*Rabbit E:* This rabbit was killed 48 hours after inoculation. At that time an early but definite keratitis in the right eye was evident, whereas the linear scratches on the left cornea presented but a negligible inflammatory reaction.

Histopathological examination showed increased stratification of the epithelium along each line of scarification and an infiltration of mononuclear and polymorphonuclear cells. No evidence for specific virus infection was discovered.

*Rabbit F:* This rabbit was killed 48 hours after inoculation. The findings were essentially the same as for rabbit A, except that examination revealed numerous Guarnieri bodies. No evidence for specific infection by herpes virus was found.

*Rabbit G:* This rabbit, killed 72 hours after inoculation, showed an extensive keratitis in the right eye, but no visible reaction in the left cornea. Serial sections were prepared from the right cornea. Examination of these sections revealed extensive epithelial proliferation at the sites of scarification, cellular infiltration, and, at one site, vesicle formation. Numerous Guarnieri bodies and a few intranuclear inclusions were seen. The two types of inclusion bodies occurred as separate groups at different sites along the lines of scarification except in a single place where both types were present in adjacent cells and in the same cell (Fig. 4).

The results of Experiment 3 showed that vaccinia virus and herpes virus in combination can simultaneously infect a single cell, for in one of the three host animals both an intranuclear inclusion body and a cytoplasmic inclusion body were present within a single cell.

*Simultaneous Infection of the Skin and Testicle with Virus III and Vaccinia Virus*

Experiment 4 was planned to determine whether cells other than those of the anterior cornea would lend themselves to simultaneous infection by several viruses. Normal rabbit skin and testicle were used to provide host cells for the inoculation of two viruses,—virus III and vaccinia virus. Virus III, the pathogenicity of which had been enhanced by repeated testicular passage immediately before use, was selected as the virus to bring about the formation of intranuclear inclusion bodies, and vaccinia virus, which had been successfully used on previous occasions, was again utilized in an attempt to produce readily recognizable cytoplasmic inclusion bodies.

*Experiment 4.*—Two male domestic rabbits, rabbits H and I, were used. The virus mixture consisted of a 1 per cent testicular suspension containing vaccinia virus mixed with equal parts of a 10 per cent testicular suspension containing virus III. Each of four successive decimal dilutions ( $10^1$  through  $10^4$ ) of this mixture was used in 0.2 ml. amounts for endermic inoculation at four sites and for the injection of the four testicles of the two rabbits, 0.25 ml. being injected into each testicle.

When killed 4 days after inoculation, both rabbits showed definite inflammatory reactions at each site of injection. Of the eight skin sites injected, the histopathological findings of interest were confined to a single lesion which is the only one that will be considered here. This lesion resulted from the injection of the  $10^2$  dilution. It showed a few cells which contained Guarnieri bodies. These cells were surrounded by areas of necrosis, but there was little evidence of cellular infiltration. The testicles revealed numerous necrotic areas interspersed among apparently normal tissues. These areas were attributed to the effects of infection by vaccinia virus. Sections prepared from the testicles that had received the  $10^2$ ,  $10^3$ , and  $10^4$  dilutions of the virus mixture revealed intranuclear inclusion bodies of type A in the interstitial cells, a reaction characteristic of virus III infection.

No evidence of simultaneous infection of a single cell by both viruses was found.

Our failure to demonstrate multiple virus infection of single cells in this experiment might conceivably be attributed to the use of cells less suitable to the development of inclusion bodies than those of the cornea, or, and this seems more probable, the necrotizing activity of the vaccinia virus may have prevented, or at least masked, the intracellular reaction characteristic of virus III infection.

*Simultaneous Infection of Tissues Immune to Myxoma Virus by Three Viruses: Myxoma Virus, B Virus, and Vaccinia Virus*

Experiment 5 was planned in a further attempt to demonstrate multiple virus infection of cells other than those of the cornea, the rabbit papilloma, and the developing chick embryo (6). Cottontail rabbits were utilized which had been rendered hyperimmune to myxoma virus by repeated reinoculation with large amounts of it. Three viruses were introduced simultaneously: myxoma virus, to bring about an immediate and heightened local response, and vac-

cinia and B viruses, capable of producing, when together, both cytoplasmic and intranuclear inclusions within single cells. The following experiment was carried out.

*Experiment 5.*—Ten per cent suspensions of myxoma, B, and vaccinia viruses were prepared and mixed in equal parts. Portions of the resulting mixture, undiluted and diluted 1 to 10, were used in 0.5 ml. amounts for the infiltration of a skin site and of one testicle on each of two cottontails known to be hyperimmune to myxoma virus.

*Rabbit J:* This rabbit was killed 4 days after inoculation. The cutaneous sites of inoculation showed a marked inflammatory reaction with central necrosis and surrounding edema. The testicles, which were twice the normal size, were also markedly inflamed and necrotic. Examination of sections revealed that all evidence of specific virus activity was limited to the cutaneous area that had received the 10 per cent dilution. In this area, intranuclear inclusion bodies of type A and Guarnieri bodies were seen, thus establishing the presence of both B and vaccinia viruses. In no single cell, however, were both cytoplasmic and intranuclear inclusion bodies noted. All the sections of testicular tissue revealed an extensive necrotizing inflammatory reaction, but no evidence for specific virus activity. Moreover, in no instance were inclusions of the type which often accompany infection by myxoma virus found.

*Rabbit K:* This rabbit, which was killed 5 days after inoculation, showed a much more extensive inflammatory reaction than rabbit J. This was evidenced by generalized polymorphonuclear invasion and abscess formation. There was a complete absence of inclusion bodies suggestive of virus activity.

It is not surprising that detectable inclusion bodies were not present following the simultaneous introduction of the three viruses, *viz.* myxoma, B, and vaccinia viruses, when the extensive necrosis which occurred at the sites of injection is taken into consideration. The usual necrobiotic effects of B and vaccinia viruses undoubtedly were so exaggerated by the accelerated and heightened response of the hyperimmune tissues to reinjection with myxoma virus that any inclusion bodies were obscured or destroyed. Moreover, the tissues infected in this experiment were the same as in the preceding experiment in which we also failed to demonstrate multiple virus infection of individual cells.

#### DISCUSSION

The present observations establish the fact that a single epithelial cell can be simultaneously infected by two viruses. The evidence is found in the repeated demonstration that the epithelial cells of the rabbit's cornea can respond to two viruses (of which one has the capacity of forming inclusion bodies within the nucleus, the other of producing them within the cytoplasm) by the formation of both intranuclear and cytoplasmic inclusion bodies within individual cells.

Anderson (6), moreover, by utilizing different combinations of viruses corroborated our earlier findings (1, 5) and procured results similar to those reported in the present paper. She established cytological evidence for the

dual parasitization of single chick embryo cells by two viruses that yield distinctively different inclusion bodies. The combinations of viruses used were fowl pox and laryngotracheitis, fowl pox and herpes simplex, herpes simplex and vaccinia, and herpes simplex and rabies. It is of interest that experiments involving the combination of fowl pox and vaccinia, two viruses that result in cytoplasmic inclusion bodies, were unsuccessful.

From the findings set forth, it is plain that the results of the present investigations substantiate our previous studies (1). The observations made in the earlier work led us to conclude that coexistent virus infections had been produced in the cells of a virus-induced tumor, Shope's rabbit papilloma. This conclusion was based on experimental evidence that the inoculation of a mixture of two viruses into a papilloma had resulted in the presence within single cells of two sorts of inclusion bodies, cytoplasmic and intranuclear, characteristic of the viruses respectively. Since all of the cells of an actively growing papilloma presumably contain papilloma virus, the presence of these inclusions would seem to indicate the coexistence of three viruses within an individual cell.

As previously stated it has been often assumed that a single cell can be infected by but a single virus. This assumption has been based on the intimate type of parasitism that characterizes viruses—on the belief that the presence of one virus within a cell would so alter the characteristics of that cell that another virus could not be active there at the same time. This belief is no longer tenable, at least for some of the viruses with which Anderson (6) and ourselves have worked. The question remains valid, nevertheless, as concerns the viruses that have been implicated in the phenomenon known variously as "sparing effect," "interference," or "cell blockade," a situation in which one strain of a virus modifies or prevents infection by a second, usually more pathogenic, strain. Some workers have assumed that the "interfering" virus exerts an "antagonistic" effect on the other virus, thereby protecting the host cell against the more serious infection. This hypothesis first found support from experimental studies with plant viruses. These investigations (7-9) suggested that closely related strains of a single virus could not simultaneously occupy the same plant tissue. Since this antagonism did not concern immunologically distinct viruses, the phenomenon might represent a rapidly acquired specific resistance induced by related strains of virus, resulting in an infection with modified or slight manifestations. Moreover, the demonstration of one virus in the presence of resistance to a second, more pathogenic but related, virus does not necessarily mean that all of the cells are occupied by the first virus to the exclusion of the second. It is equally possible, perhaps probable, that a carrier state may coexist with a state of acquired immunity. This explanation might apply where the "interference" phenomenon has been observed in infections with animal viruses (10-19). In four instances involving different viruses (12,

13, 15, 19), however, the manifestations of an otherwise serious virus disease were obscured after two apparently unrelated viruses had been inoculated within a few hours.

Many hypotheses have been advanced to explain the intimate type of parasitism characteristic of so many virus infections, to account for the sharply defined host specificity and tissue affinity so frequently observed, and to make understandable the mechanisms whereby a virus can multiply within its host cell. These hypotheses have been commonly predicated on the "lock and key" concept that the virus "fits" into the enzyme systems of the host cell, perverting the cell's metabolism, such perversion resulting in the various manifestations of deranged cellular activity and cellular destruction characteristic of most virus infections. It is reasonable to suppose that when two or more viruses simultaneously exhibit their characteristic activities within a single cell, they "fit" into the intracellular mechanisms at different "points;" whereas when they compete for the same "point," the "blockade" or "interference" phenomenon is observed. Many analogies from the fields of enzymology, pharmacology; and immunology come immediately to mind. One may perhaps hopefully expect that further study of multiple virus infections of single cells will help in elucidating not only the phenomenon of "interference," but also the nature of intracellular virus activity.

#### SUMMARY

Evidence is presented to show that two or more viruses can simultaneously manifest their characteristic activities within individual epithelial cells of the normal rabbit's cornea. This evidence, together with that previously presented (1, 5, 6), makes plain that multiple virus infection of a single host cell can take place in corneal cells, in the cells of chick embryos, and in those of rabbit tumors, both benign (Shope's papilloma) and malignant.

Certain implications of the findings are discussed.

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## EXPLANATION OF PLATES

### PLATE 15

The photographs were made by Mr. Merwyn C. Orser from sections stained by Giemsa's method.

FIG. 1. Portion of a section of cornea from rabbit A originally inoculated with a mixture of vaccinia virus and B virus in suspension. The cell designated by the arrow shows clearly the presence of a type A intranuclear inclusion body of B virus infection and a cytoplasmic inclusion body of vaccinia virus infection.  $\times 1500$ .

FIGS. 2 and 3. Section of cornea from rabbit C which was killed 72 hours after a mixture of vaccinia virus and B virus was inoculated into the scarified cornea. Two cells well separated from all adjacent cells are shown. Within each of these cells will be seen a Guarnieri body and a type A intranuclear inclusion body. By using an oil immersion lens and focusing at four successive levels, four photographs were taken (*a*, *b*, *c*, and *d* of Fig. 3), which show unequivocally the singleness of each cell and the presence within each cell of both a cytoplasmic inclusion body and an intranuclear inclusion body. Fig. 2,  $\times 1500$ ; Fig. 3,  $\times 1250$ .

FIG. 4. Section of cornea from rabbit G which was killed 72 hours after inoculation with a mixture of vaccinia virus and herpes virus. A Guarnieri body and a type A intranuclear inclusion body within a single cell are indicated by arrows.  $\times 1500$ .



(Syverton and Berry: Multiple virus infection of single cells)

## PLATE 16

These prints were made by Mr. Adrian Ter Louw and Mr. Charles Brownell who employed a modification of the Kodak wash-off relief process for separation negatives that were exposed directly, from sections stained by Giemsa's method.

FIG. 5. The two cells depicted in Fig. 3 are shown in color. The three photographs were made by using an oil immersion lens and focusing at three successive levels.  $\times 1500$ . Fig. 5 *a* shows the type A intranuclear inclusion bodies in sharp focus; Fig. 5 *b*, an intranuclear inclusion and a cytoplasmic inclusion body in each cell but none of the inclusion bodies in sharp focus; Fig. 5 *c*, the granular cytoplasmic inclusion bodies are in sharp focus.

FIG. 6. Sections of cornea from rabbit D which was killed 72 hours after inoculation to show in color type A intranuclear inclusion bodies (Fig. 6 *a*) and cytoplasmic inclusion bodies (Fig. 6 *b*).  $\times 600$ .





(Syverton and Berry: Multiple virus infection of single cells)



# THE INFLUENCE OF INJECTIONS OF HOMOLOGOUS HEMOGLOBIN ON THE KIDNEYS OF NORMAL AND DEHYDRATED ANIMALS

By JOSEPH J. LALICH, M.D.

(From the Department of Pathology, University of Wisconsin  
Medical School, Madison)

PLATE 17

(Received for publication, April 11, 1947)

The importance of intravascular hemolysis following mismatched transfusions of whole blood is generally appreciated. The probability of intravascular hemolysis has been greatly enhanced with the accentuated employment of whole blood in the treatment of patients. Lucké, who quotes Mallory and Angevine and Harman, states that the incidence of lower nephron nephrosis in battle casualties is 15.2 to 18.6 per cent (1). Although hemoglobin was suspected to be a causative factor in many of these cases, attempts to produce experimental nephrosis with hemoglobin have resulted in unexplained difficulties.

Yorke and Nauss were the first to report suppression of urine following intravenous injections of hemoglobin into rabbits (2). Baker and Dodds confirmed this and ascribed the precipitation of the hemoglobin in the tubules to an acid urine (3). DeGowin, Warner, and Randall injected homologous hemoglobin into dogs and reported obstruction of tubules close to Henle's loop in 6 of 18 of their animals (4). These authors attributed the obstruction to an acid urine. While attempting to reproduce hemoglobininuric nephrosis in rabbits, DeNavasquez was unable to confirm the results of the previous workers (5). Hueper was unable to detect any alteration in the kidneys of dogs following injections of hemoglobin (7). Bing found hemoglobin ineffective in the production of renal failure in normal and acidotic dogs (6). Yuile, Gold, and Hinds were able to produce precipitation of hemoglobin in the tubules of rabbits, following intravenous injection of hemoglobin, only after previously clamping the renal artery or injecting sodium tartrate (8).

The recent literature therefore indicates that solutions of pure hemoglobin can be injected intravenously into animals with either acid or alkaline urine without producing significant impairment of renal function. Studies (4, 8) also suggest that some factor or factors must precede or be associated with the hemoglobinemia if renal impairment is to develop. In view of this probability it was decided to study the influence of injections of homologous hemoglobin in normal and dehydrated animals.

## Method

*Animals.*—The animals used in this study were Sprague-Dawley rats weighing 71 to 110 gm., guinea pigs weighing 155 to 680 gm., and albino rabbits weighing 1.8 to 3.0 kg. Both male and female animals were used.

*Preparation of Hemoglobin.*—Blood was withdrawn from the heart of several animals and pooled in a flask containing sodium citrate (5 mg./ml.). The blood was centrifuged at 2500 R.P.M. for 1 hour, the plasma withdrawn, and the erythrocytes washed with an equal volume of physiologic saline solution. Following the second centrifugation the saline was removed and the erythrocytes were laked by the addition of two volumes of recently distilled water. The stroma was removed by 45 minutes of centrifugation at 15,000 R.P.M. and subsequent filtration through two thicknesses of Whatman's No. 40 filter paper at a negative pressure of 500 to 700 mm. Hg. The hemoglobin concentration was determined in a 1:300 dilution of 0.1 per cent  $\text{Na}_2\text{CO}_3$  solution with a photoelectric colorimeter. Hemoglobin solutions, never older than 2 days, were kept at 4–6°C. when not in use.

*State of Hydration and Method of Injection.*—The rats and guinea pigs were fed and offered water up to the time of injection. Hemoglobin solutions containing stroma were injected intraperitoneally in single doses into these animals.

Water was withheld from the rabbits for periods of 1 to 5 days. Stroma-free hemoglobin solutions were injected intravenously at one time, or in divided doses on successive days toward the end of the dehydration period. Water was then withheld for a period of 18 to 24 hours after the last injection. Thereafter 200 ml. of water was given daily to 6 rabbits (Nos. 1 to 6) for 1 week. One rabbit (No. 7) received 28 ml. daily for 7 days. The last 9 rabbits (Nos. 8 to 16) received 25 ml./kg. of water for 1 week. After the 14th day the rabbits were given food and water at desire.

*Blood Studies.*—Heart blood was withdrawn at intervals for non-protein nitrogen determinations and hematocrit studies.

*Subsequent Observations.*—The rabbits were weighed both before and following dehydration as well as at the time of necropsy. Animals which did not die following the injections were killed from 4 to 40 days thereafter. Autopsies were performed, the tissues fixed in 10 per cent formalin, sectioned, and stained with hematoxylin and eosin.

### *Results of the Injections into Rats and Guinea Pigs*

The rats received hemoglobin suspensions in amounts of 5, 6, and 7 gm./kg. of body weight intraperitoneally. Following the injection of 5 gm./kg. one of 11 animals died on the 2nd day. After 6 gm./kg. one of 12 animals died after 24 hours. When 7 gm./kg. were given, 9 rats died between 12 to 24 hours; 2 died after 24 hours and only one survived. Practically all of the animals had hemoglobinuria. Those that died developed cyanosis about the jaws and feet. Their abdomens remained distended following the injections, and at autopsy there was peritoneal fluid containing hemoglobin. On gross inspection of the organs no significant change was noted than cyanosis. Autopsies made on the animals which survived revealed no abnormalities. Microscopic examination of the kidneys revealed an occasional cast in only 2 rats. No casts or tubular changes were observed in sections of the remaining 35 rats.

The guinea pigs received 1, 2, 3, and 3.5 gm./kg. of hemoglobin intraperitoneally in one dose. Six animals which received 1 gm./kg. survived without any ill effects. One of 6 guinea pigs died after an injection of 2 gm./kg. Following the injection of 3 gm./kg., 3 died after 12 to 24 hours; one died on the 3rd day from peritonitis, and 2 survived. In a final group 3.5 gm./kg. produced death in all of 8 guinea pigs in less than 10 hours. Those which died following the injections usually had minimal traces of hemoglobin in the peritoneal fluid. Sections from these 26 animals did not reveal any casts or tubular changes.

### *Results of the Injections into Rabbits*

The results obtained with dehydration and intravenous injections of hemoglobin into rabbits are tabulated in Table I. It illustrates the influence of de-

hydration on the precipitation of hemoglobin in the tubules. The NPN was elevated in 8 of the 14 recorded instances. In 5 the elevation was temporary; in 3 rabbits, which subsequently died in uremia, the NPN was in excess of 199 mg. per cent; in 6 rabbits the NPN remained below 43 mg. per cent.

TABLE I

*The Influence of Dehydration on the Production of Hemoglobinuric Nephrosis Following Intravenous Injections of Hemoglobin into Rabbits*

No.	Dehydration	Quantity of blood removed during Dehydration	Quantity of hemoglobin injected	Highest recorded NPN	Interval after injection	Total weight of kidneys	Microscopic examination of kidneys		
							Pigment casts	Dilated tubules	Necrosis of tubular epithelium
	days	ml.	gm./kg.	mg. per cent	days	gm.			
1	1	0	1.9(1)1	—	14	—	1+	—	—
2	1	0	1.3(1)1	—	1*	—	—	—	—
3	4	12	1.2(5)2†	61	29	17.5	2+	+	—
4	4	19	1.5(5)2	67	6	17.5	2+	+	—
5	4	49	1.4(4)2	42	40	20.5	—	+	—
6	4	69	1.0(3)2	29	1*	15.1	1+	—	—
7	3	12	1.6(4)2	35	40	19.0	2+	+	—
8	3	12	1.5(6)2	300+	11*	26.0	3+	+	+
9	3	12	1.6(6)2	30	2*	—	2+	+	—
10	3	12	1.6(6)2	34	4	15.2	1+	+	+
11	5	17	1.7(7)3	63	15	22.6	3+	+	—
12	5	38	1.8(7)3	200	14*	19.2	3+	+	+
13	5	42	1.7(7)3	97	14	12.8	2+	+	+
14	5	34	1.7(7)3	60	15	15.4	2+	+	—
15	4	31	1.5(8)2	300+	6*	36.0	3+	+	+
16	4	54	1.4(6)2	42	14	14.8	1+	+	—

1+ = less than one cast per low power field.

2+ = 1 to 5 casts per low power field.

3+ = 5 to 15 casts per low power field.

\* = died.

† 1.2(5)2 = 1.2 gm./kg. injected in five divided doses on 2 successive days.

### *Pathological Findings.—*

*Kidneys.*—When death occurred prior to the 4th day no significant alterations were noted in any of the organs. The kidneys appeared congested. After the 4th day in practically all animals there were minute dark brown flecks 1 to 2 mm. across on the surface of the kidney. The brown pigment accentuated the cortical striations on the cut surface and was principally deposited in the cortex of the kidney (Fig. 1.). There were wide variations in the amount of pigment, but its localization was uniform. The combined weight of the kidneys in each instance exceeded the normal median value of 12.5 gm. reported by Brown, Pearce, and Van Allen (9).

The casts were orange or green yellow in color and therefore easily distinguishable from eosin-staining casts, usually considered to consist of albumen. Because the chemical nature

of the casts in the tubules remains undetermined, they will be referred to as pigment casts. Practically all of the pigment casts were localized in the cortical area, principally in the distal convoluted tubules. A few casts were also found in Henle's loops and the collecting tubules (Fig. 2 and 3). Prior to the 4th day the substance observed in the lumina was reddish yellow and of a homogeneous glass-like consistency. In view of the lack of granularity, the pronounced yellow color, and the absence of associated tubular changes, this substance as occurring in three animals was not considered to represent formed casts. Excluding these animals, however, characteristic pigmented casts were demonstrated in 12 of 13 rabbits from 4 to 40 days after the injections of hemoglobin. In conjunction with the presence of casts there was an associated tubular dilation. In the majority of instances there were focal areas of lymphoid infiltration. In only 5 of 13 kidneys was there minimal necrosis of tubular epithelium. Necrosis of the tubular epithelium and lymphoid infiltration appeared to follow rather than precede the plugging of tubules by the pigmented casts.

*Heart.*—The hearts in some instances showed minimal pericardial hemorrhages incident to cardiac puncture. Microscopic examination revealed extensive areas of focal necrosis and calcification of myocardial fibers in 2 animals.

*Lung.*—Three of the animals had an associated pneumonia at the time of death. Microscopic examination did not reveal any consistent variations from normal.

*Liver.*—Gross examination of the liver revealed no significant change. Microscopic examination revealed minimal collections of pigment about the central vein in only one animal.

*Spleen.*—The spleen was not enlarged in any instance. Microscopic examination usually showed moderate phagocytosis of hemosiderin by macrophages.

#### DISCUSSION

In accordance with previous findings in animals (5, 6), the experiments on rats and guinea pigs clearly indicate that in these animals on a suitable diet with adequate quantities of water, hemoglobin is readily excreted by the kidneys. Only a few casts were demonstrable in 2 of 63 animals after injections of large amounts of hemoglobin. Several features become evident in the findings in rabbits. Apparently a certain time must elapse before the intratubular hemoglobin is precipitated or sufficiently concentrated to occur in casts that are demonstrable in tissues by the usual staining methods. Furthermore a relationship exists between the quantity of hemoglobin injected and the state of hydration of the animal. Yorke and Nauss (2) observed casts after injecting larger doses of hemoglobin (2.5 to 41.0 gm.) over periods of 1 to 5 days. In the present study we were able to demonstrate casts in the tubules consistently, after injections of relatively small quantities of hemoglobin (1 to 1.9 gm./kg.) if the previous intake of water had been restricted.

It is difficult to maintain rigid control of all the variables in an experiment of this type. Following the withholding of water some rabbits stopped eating immediately, whereas others continued to eat for 2 to 3 days. After identical periods of dehydration, quantitative differences were observed in weight loss, the volume of urine excreted, and in responses to intravenous injections of hemoglobin. Intravenous hemoglobin injections were without any apparent immediate effect in most rabbits; but some became extremely weak and a few

died following injections. It seems doubtful that the toxic manifestations exhibited by these animals were due to potassium salts; however, such a possibility has not been excluded.

As far as we have been able to determine, consistent reproduction of hemoglobinuric nephrosis has not heretofore been accomplished without a preliminary direct trauma to the kidneys. It is now apparent that such a lesion can be produced consistently in rabbits when dehydration precedes the intravenous injections of hemoglobin. Whether dehydration exerts a direct influence on the precipitation of pigment casts or acts indirectly through altered physiologic responses, remains to be determined. In view of the present findings it is felt that similar studies should be made in other species. Whether the relationship of dehydration and hemoglobinemia to the production of hemoglobinuric nephrosis is peculiar to the rabbit or exists in other species, particularly man, is a problem of large importance.

#### SUMMARY

1. Dehydration, if sufficiently prolonged, favors the accumulation of hemoglobin or its derivatives in the kidneys of rabbits, principally casts formed in the distal convoluted tubules.
2. Once pigment casts have been produced in the distal convoluted tubules, there is obstruction, with atrophy and dilatation of the tubules proximal to the obstruction.
3. When the involvement is sufficiently extensive, there is elevation of the non-protein nitrogen and some animals die because of renal failure.

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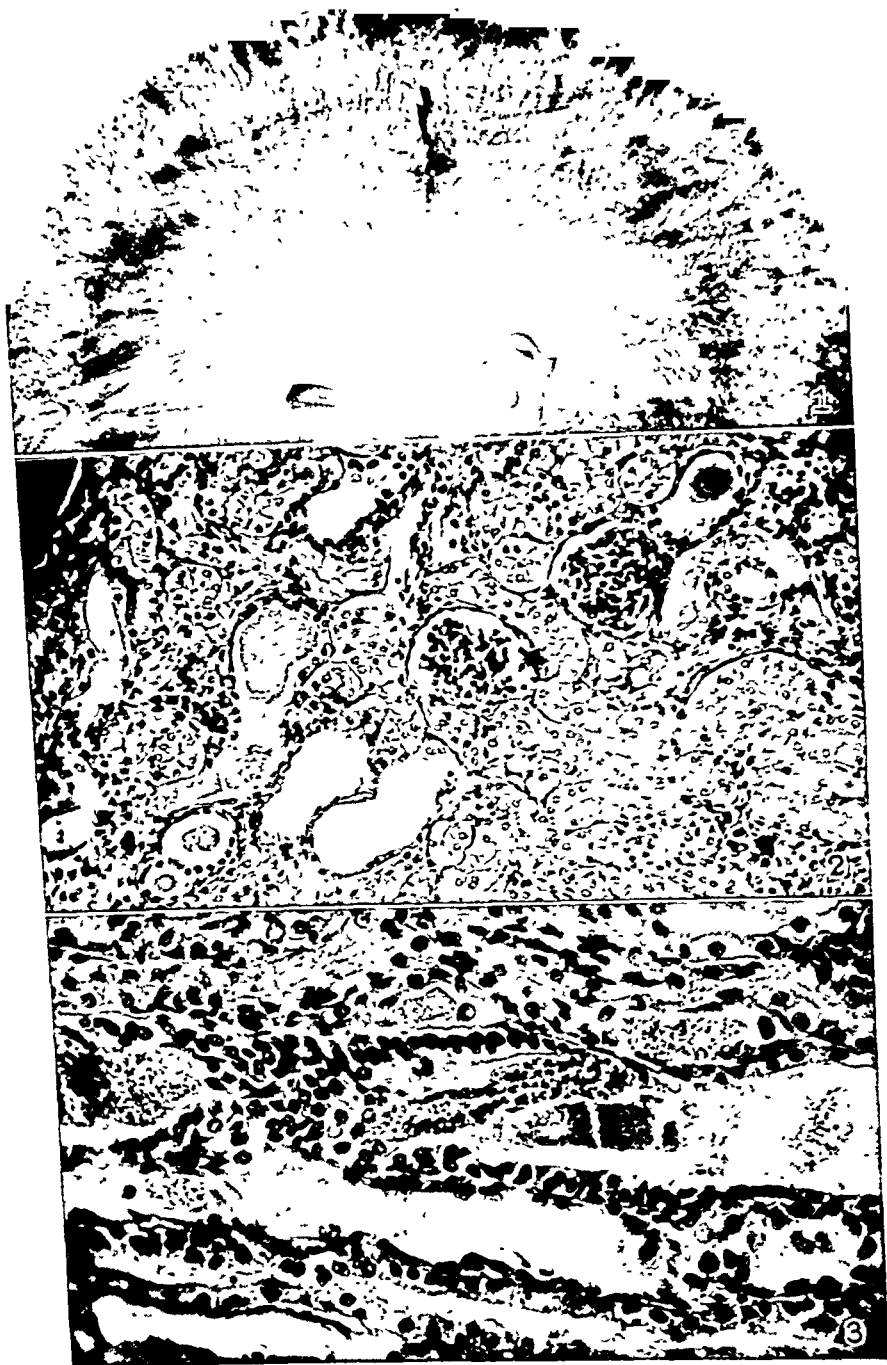
## EXPLANATION OF PLATE 17

FIG. 1. Hemisection of kidney, rabbit 12. Water was withheld for 5 days and then the rabbit was given 1.8 gm./kg. of hemoglobin in seven doses during the last 3 days. It died in uremia 14 days after the initial injection. Large collections of dark brown pigment are present, localized principally in the cortex, and minimal quantities can be seen in the medulla.  $\times 3$ .

FIG. 2. Kidney, rabbit 12. There are pigment casts in the distal convoluted tubules and in Henle's loops. There is an associated tubular dilatation with flattening of epithelial cells. Hematoxylin and eosin stain.  $\times 150$ .

FIG. 3. Kidney, rabbit 12. The granularity of the pigment casts is a typical feature. There are, in addition, dislodged epithelial cells filling one of the tubules. Hematoxylin and eosin stain.  $\times 250$ .





(Lalich: Homologous hemoglobin injections and kidneys)



# INFECTION OF MICE WITH MAMMALIAN TUBERCLE BACILLI GROWN IN TWEEN-ALBUMIN LIQUID MEDIUM

BY CYNTHIA PIERCE, Ph.D., RENÉ J. DUBOS, Ph.D.,  
AND GARDNER MIDDLEBROOK, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATE 18

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Cultures of pathogenic mycobacteria growing rapidly and diffusely in liquid media containing serum albumin and a synthetic water-dispersible ester of oleic acid are extremely virulent for chick embryos, mice, guinea pigs, and rabbits (1, 2). The present paper deals with some of the factors which affect the course and outcome of the experimental disease produced in mice by the injection of mammalian tubercle bacilli cultivated in this manner.

## Materials and Methods

*Cultures.*—Several strains of human and bovine tubercle bacilli of various degrees of virulence were used in the course of the present study. For the sake of brevity, the results will be illustrated chiefly with data obtained with two cultures derived from the same strain, H37Rv (virulent) and H37Ra (avirulent).<sup>1</sup>

*Medium.*—Cultures of mammalian tubercle bacilli growing diffusely in aqueous solution and exhibiting a high degree of pathogenicity were obtained in a variety of synthetic media to which were added small amounts of the water-dispersible ester of oleic acid (Tween 80)<sup>2</sup> (0.05 to 0.1 per cent) and of bovine serum albumin, fraction V<sup>3</sup> (0.1 to 0.5 per cent). Most experiments were carried out with a medium of the following composition:

	gm.
KH <sub>2</sub> PO <sub>4</sub> .....	1.0
Na <sub>2</sub> HPO <sub>4</sub> ·12 H <sub>2</sub> O.....	6.5
CaCl <sub>2</sub> .....	0.0005
MgSO <sub>4</sub> ·7 H <sub>2</sub> O.....	0.001
ZnSO <sub>4</sub> .....	0.0001
CuSO <sub>4</sub> .....	0.0001
Ferric ammonium citrate.....	0.05
Asparagine.....	2.0
Enzymatic digest of casein.....	2.0
Tween 80.....	0.5
H <sub>2</sub> O.....	1000.0

<sup>1</sup> These cultures were obtained through the courtesy of Mr. William Steenken, Jr., from the National Tuberculosis Association Standard Culture Depot at Trudeau, in the spring of 1946.

<sup>2</sup> Tween 80 was generously supplied by the Atlas Powder Company, Wilmington, Delaware.

<sup>3</sup> Bovine plasma fraction V (serum albumin) was obtained from the Armour Laboratories, Chicago, Illinois.

The medium, adjusted to pH 6.8, was distributed in 5 cc. amounts into Pyrex test tubes (25 mm. diameter) and autoclaved at 17 pounds pressure for 15 minutes. To each tube was then added 0.2 cc. of 5 per cent bovine albumin (plasma fraction V) and 0.05 cc. of a 50 per cent glucose solution (autoclaved in distilled water). Details concerning the preparation and properties of the medium are described elsewhere (3, 4).

*Maintenance of Cultures.*—Stock cultures were maintained by inoculating 0.1 cc. of a 7 day old culture into 5 cc. of medium, and incubating at 37.5°C. for 7 days. At the end of the incubation period, the growth appeared as a dense deposit in the bottom of the tube. Gentle agitation gave rise to a fine and fairly stable bacterial suspension which seemed homogeneous on macroscopic examination although microscopic study showed it to consist chiefly of small clumps of bacilli. These cultures contained 0.15 to 0.2 mg. of bacilli (dry weight) per cc. of medium (corresponding to  $10^8$  to  $10^9$  organisms). More detailed description of their characteristics will be presented in the following publication (5).

The virulent cultures were frequently passed through mice and reisolated from infected lung, heart, or spleen tissue. The organ utilized was removed aseptically, and ground with sand in a sterile mortar in the presence of an equal volume of 0.5 per cent bovine albumin solution in distilled water. Serial dilutions of this suspension were inoculated in 0.5 cc. amounts into tubes containing 5 cc. of liquid medium. In the case of H37Rv, heavy growth was usually obtained in the tubes inoculated with the  $10^{-4}$  and  $10^{-5}$  dilution of infected tissue suspension after 10 to 14 days' incubation at 37.5°C. The culture was then transferred to new medium for use as an infective inoculum. Virulent cultures can also be readily recovered by inoculation of 5 cc. of medium with a loopful of mouse brain tissue removed from animals infected by the intracerebral route. In general, cultures were discarded after 8 to 10 transfers in liquid medium, and isolated again from infected mice.

*Preparation of Bacterial Suspension in Egg Yolk.*—Fresh yolk was removed aseptically from a hen's egg and diluted with an equal volume of 0.85 per cent saline. One part of the suspension was added to an equal part of the bacterial culture and the mixture emulsified by vigorous pipetting just prior to inoculation. It is interesting to note that the presence of Tween 80 (0.05 per cent) greatly facilitates the preparation of a fine emulsion of egg yolk, thus preventing the formation of emboli as a result of intravenous injection.

*Maintenance of Mice.*—Mice 3 to 5 weeks old of a number of strains were obtained from the breeding colonies of the Rockefeller Institute and from some commercial farms. They were raised and kept in a room free from tuberculous animals until the time of infection. Immediately after this they were transferred to glass jars bedded with cedar shavings and covered with wire mesh tops, 4 or 6 mice per jar, in a room continuously irradiated with ultra-violet light. They were fed once daily a diet of white bread and milk. There was no obvious evidence of mouse typhoid, or of any infectious disease (other than tuberculous infection in the inoculated animals) during the course of the experiments to be reported.

The mice were weighed once a week, the 4 or 6 animals in each jar (or their survivors) being weighed as a unit. They were transferred to clean jars with new bedding at that time.

Records were made of dead animals every morning, and autopsies performed on them as well as on all surviving animals at the end of the experiments (3 to 6 weeks after infection).

## RESULTS

### *1. Infection of Mice by the Intravenous Route*

The finely dispersed state of tubercle bacilli growing diffusely in Tween 80-albumin liquid medium permits direct introduction of these cultures into the blood stream of experimental animals. This eliminates the necessity

of mechanical trituration of the culture, the usual practice with surface growths obtained on solid egg media or as pellicles on liquid media. The following experiment was instituted to determine the pathogenicity of mammalian tubercle bacilli for Swiss albino and for line 1 dba mice.

Swiss albino mice, 4 weeks of age (average weight 17 gm.), and line 1 dba strain mice, 4 weeks of age (average weight 14 gm.), were inoculated by the intravenous route with amounts of H37Rv culture in Tween-albumin medium ranging from 0.02 to 0.00003 cc., diluted to a final volume of 0.1 cc. with sterile medium. All animals surviving for 39 days after infection were sacrificed then and observations were made as to the presence, extent, and character of pulmonary disease. The results are presented in Table I.

TABLE I

*Response of Swiss Albino and Line 1 dba Mice to Infection with Mammalian Tubercle Bacilli via the Intravenous Route*

Mouse strain	Culture H37Rv	No. mice	Death and survival	Macroscopic appearance of lungs of surviving animals
	cc.			
Swiss albino	0.02	6	D25, D33, S, S, S, S	Small, discrete, non-elevated* gray areas Entire surface of lungs flat
	0.003	6	S, S, S, S, S, S	
	0.0003	6	S, S, S, S, S, S	
	0.00003	6	S, S, S, S, S, S	
Line 1 dba	0.02	6	D5, D27, D27, S,* S,* S*	Large, confluent, elevated glistening, grayish-white nodules, occasionally involving whole lobes
	0.003	6	D30, D32, D36, D36, S,* S*	
	0.0003	6	S, S, S, S, S, S	
	0.00003	6	S, S, S, S, S, S	

D= death; the numeral indicates the number of days after infection at which death occurred.

S= survival for a period of 39 days at which time all surviving animals were sacrificed.

\* These animals were extremely emaciated at the end of the experiment and would have probably died within a few days; in all subsequent experiments dba mice infected with 0.01 cc. of culture died within 3 weeks after infection.

As indicated in Table I, intravenous injection of tubercle bacilli resulted in detectable pulmonary lesions in all animals, however small the infective dose. It is obvious, however, that the disease was much more severe in the line 1 dba than in the Swiss albino mice. Only two of the albino animals died of infection with the largest infective dose (0.02 cc.) and the others appeared healthy and were gaining weight when the experiment was terminated. A much smaller infective dose (0.003 cc.) caused higher mortality of the line 1 dba mice; moreover, the surviving animals of this strain were so emaciated when sacrificed that it is unlikely that they would have survived for many more days. Subsequent experience with larger numbers of experimental

animals has repeatedly confirmed the trend of the observations reported in Table I. In general, line 1 dba mice infected intravenously with 0.1 cc. of culture die within 1 week, too soon to show gross pulmonary lesions; infection with 0.01 cc. of culture usually results in death within 3 weeks, with extensive involvement of lung tissue.

In all of the strains of mice tested, 22 in all, the lungs of animals dying within the first 2 weeks after infection showed as a rule many hemorrhagic areas 1 to 2 mm. in diameter whereas pearly gray lesions characterized the later stages of the disease. Although the histopathology of experimental tuberculous infections in mice will be described elsewhere, it may be useful to present at this time a short statement of some of our preliminary observations:—

Histological sections of the lungs of mice dying 2 or more weeks after infection with mammalian tubercle bacilli revealed the presence of numerous lesions when stained by hematoxylin-eosin technique. These lesions varied in size from small collections of polymorphonuclear leucocytes and round cells filling an alveolus, to lesions involving many alveoli, characterized by a central area of necrosis and massive infiltration with inflammatory cells. Shadowy outlines of the alveolar septae were always visible even in the central area of necrosis. Distension of the alveoli at the periphery of these lesions indicated the presence of edema fluid. Parallel histological sections stained by the Ziehl-Neelsen technique revealed the presence of enormous numbers of acid-fast bacilli located both intracellularly and extracellularly. Although bacilli were usually found only in lesions in the pulmonary parenchyma, they could also be seen in some cases in the lumen of the bronchi.

Figs. 1 to 4 illustrate the type of lesions obtained in Swiss albino and line 1 dba mice.

The progress of tuberculous infection in mice can be followed not only by the development of pulmonary lesions, but also by weight losses of the infected animals.

Albino mice of the Rockefeller Institute strain 4 weeks of age and weighing 17 gm. were inoculated intravenously with 0.1 cc. or 0.01 cc. of the virulent culture, H37Rv, or with 0.2 cc. of the avirulent variant of the same strain, H37Ra. Body weights were recorded (as the average of six animals or their survivors) at weekly intervals. The experiment was terminated at the end of 4 weeks.

As appears from the results presented in Table II, weight loss became evident 2 weeks after injection of 0.1 cc. and 3 weeks after injection of 0.01 cc. of virulent H37Rv bacilli. This weight loss was not due merely to a non-specific toxic effect of the bacillary material which was unrelated to virulence, since no similar effect was observed when even larger amounts of culture of the avirulent variant H37Ra were injected, also by the intravenous route. No gross pulmonary lesions were observed in animals inoculated with the avirulent culture; further-

more, impression smears of the lungs stained by the Ziehl-Neelsen technique failed to reveal the presence of acid-fast bacilli.

TABLE II

*Response of Albino Mice of Rockefeller Institute Strain to Intravenous Inoculation with H37Rv (Virulent) and H37Ra (Avirulent) Mammalian Tubercle Bacilli*

Culture	Egg yolk suspension	Weekly weight after inoculation					No. dead/ Total
		Initial	1 wk.	2 wks.	3 wks.	4 wks.	
cc.	cc.	gm.	gm.	gm.	gm.	gm.	
H37Rv 0.1	0	18.3	20.0	17.9	15.8	Dead	6/6
H37Rv 0.01	0	18.3	21.3	22.2	19.7	19.2	5/6
H37Ra 0.2	0	18.3	21.6	24.2	25.9	26.7	0/6
H37Ra 0.2	0.1	18.3	20.9	23.3	24.6	25.3	0/6
0 0	0.1	18.4	19.8	23.6	24.2	25.3	0/6

## 2. Infection of Mice by the Intracerebral Route

The following experiment was set up to determine whether virulent tubercle bacilli injected into mice by the intracerebral route multiply in the brain tissue and can be disseminated thence to other tissues.

Swiss albino mice, 4 weeks of age and 17 gm. in weight, and line 1 dba mice, 4 weeks of age and 14 gm. in weight, were inoculated into the brain, while under ether anesthesia, with graded amounts of bacilli resuspended in a final volume of 0.03 cc. of sterile medium. At weekly intervals some of the animals were sacrificed, and examined for the presence of gross pulmonary lesions; at the same time, impression smears of the brain tissue were stained by the Ziehl-Neelsen technique and examined for the presence of acid-fast bacilli. Four strains of tubercle bacilli were used for infection: H37Rv, a classical laboratory strain of mammalian origin, Ravenel, a classical laboratory strain of bovine origin, Waller, a human strain recently isolated from sputum, and Number 3817, a bovine strain recently isolated from human pathological material. The results with all four strains were identical. They are summarized in Table III.

The results presented in Table III reveal the surprising fact that evidence of marked multiplication of bacilli could be detected within 1 week in the cerebral hemisphere of the brains infected with even a very small dose (10 to 100 organisms). But, on the other hand, it should be remarked that invasion of the other hemisphere and gross pulmonary infection were detectable only after 2 to 3 weeks. As in the case of infection by the intravenous route the extent of the pulmonary lesions resulting from intracerebral inoculation was dependent upon the strain of mouse used; the dba mice exhibited much more extensive lesions than Swiss albino animals.

Experiments are now in progress to follow more accurately by histological and quantitative bacteriological techniques the process of bacterial proliferation

within the brain tissue. Preliminary results indicate that an increase in the number of bacilli becomes evident 2 to 3 days after infection and that, at this early time, most of the bacilli appear packed within large mononuclear cells. Finally, it may be worth mentioning at the present time that tuberculous infection has been established by injecting intracerebrally 0.01 cc. of sputum obtained from tuberculous patients.<sup>4</sup>

TABLE III

*Response of Swiss Albino and Line 1 dba Mice to Intracerebral Infection with Mammalian Tubercle Bacilli*

Culture H37Rv  cc.	Presence of bacilli in brain tissue 1 wk. after infection		Pulmonary lesions 3 wks. after infection	
	Swiss mice	dba mice	Swiss mice	dba mice
0.01	+	+	++++	++++
0.0015	+	+	+++	+++
0.00015	+	+	++	++
0.00003	+	+	++	++
0.000003	+	+	+	+
0.0000003	—	+	—	+

### 3. Intraperitoneal Infection of Mice; The Effect of Egg Yolk

It was soon observed that the minimal infective doses of mammalian tubercle bacilli required for the establishment of a fatal infection are much larger (five- to ten-fold) by intraperitoneal than by intravenous injection. The results of the infection are also less regular and in particular, selective localization of the lesions in the lung tissue is a less prominent feature.

Many efforts were made to increase the virulence of the cultures by passage through mice and through chick embryos. No convincing quantitative data are as yet available concerning the effect of mouse passage on virulence. It was found, however, that direct injection of some of the contents of the yolk sac of infected chick embryos into the peritoneal cavity of mice gave rise to a rapidly fatal infection with striking pulmonary localization. This phenomenon is illustrated and analyzed in the following experiments.

Embryonated hen eggs 7 days old were inoculated with 0.1 cc. of the virulent culture H37Rv, introduced into the yolk sac. Ten days after infection, multiple lesions were present on the chorioallantoic membrane (1). The yolk sac and its contents were collected at that time and together emulsified with an equal volume of physiological saline; stained preparations

<sup>4</sup> We wish to acknowledge with thanks the cooperation of Dr. Walsh McDermott and Dr. Susan Hadley of the New York Hospital, who supplied us with selected samples of human tuberculous material.



and cultivation of this material in Tween-albumin media revealed the presence of enormous numbers of acid-fast bacilli (approximately  $10^8$  per cc.). Within 2 hours after recovery from the infected embryo, graded amounts of the diluted yolk material were injected intraperitoneally into albino mice; for the purpose of comparison, other mice of the same breed and age were inoculated with comparable numbers of bacilli grown in Tween-albumin liquid medium.

Identical results—not to be detailed here—were obtained in a large number of experiments. The bacilli in the yolk sac exhibited much higher infectivity than those recovered from culture media as measured in terms of death rate or of the extent of the gross pulmonary lesions. However, bacilli recovered from infected yolk sacs and cultivated in Tween-albumin media did not appear more virulent than the stock culture when injected into mice by any route. Further analysis of the findings was therefore undertaken in order to determine the possible influence of the egg yolk material as such upon the infectivity of tubercle bacilli.

Albino mice of the Rockefeller Institute strain, 4 weeks of age and 18 to 20 gm. in average weight, were inoculated *via* the intraperitoneal route with graded amounts of cultures grown in Tween-albumin medium. Sterile egg yolk emulsion was added to some of the inocula—as indicated in Table IV—according to the method described earlier in this report. The results of the injections are presented in Table IV in terms of weekly weight changes of the animals, numbers of deaths within a period of 3 weeks, presence and extent of gross pulmonary lesions, and enlargement of the spleen and lymph nodes.

The results presented in Table IV show that the addition of normal egg yolk to the culture of H37Rv prior to intraperitoneal injection markedly enhances the infectious process, as measured either in terms of weight loss or by death of the animals. The longer survival time was correlated with extreme splenic enlargement and small pulmonary lesions, whereas more acute disease resulted in less pronounced enlargement of the spleen, but much more pronounced pulmonary lesions. All attempts to reproduce the enhancing effect of egg yolk by injecting the material into mice either before or after introduction of the bacilli failed to modify the course of the disease. This fact is illustrated in the last line of Table IV where it is shown that addition of 0.25 cc. of diluted egg yolk 48 hours after infection resulted in extreme enlargement of the spleen, minimal pulmonary lesions, and no death within 3 weeks, this being a disease picture which could not be differentiated from that obtained with bacilli alone.

Further evidence of the increase in infectivity obtained by addition of egg yolk to the inoculum is illustrated in Table V. This enhancing effect is not due to a non-specific toxic action of the egg yolk since addition of this material to larger doses of the avirulent variant of H37 (H37Ra) failed to affect the weight curves of the inoculated animals, or to cause in them any evidence of disease.

The enhancement of infectivity of virulent tubercle bacilli by means of egg

TABLE IV

*Effect of Egg Yolk upon Infectivity of Mammalian Tubercle Bacilli for Rockefeller Institute Albino Mice*

Culture H37Rv (i.p.)	Egg yolk suspension	Weekly weight changes				Average spleen weight	Gross pathological findings Macroscopic appearance of tissues	No. dead/ Total
		Initial	1 wk.	2 wks.	3 wks.			
cc.	cc.	gm.	gm.	gm.	gm.	mg.		
0	0	18.8	19.4	20.8	21.8	97	Normal	0/6
0	0.25	20.0	19.9	20.5	22.5	75	Normal	0/6
1.0	0	21.0	20.3	21.7	22.6	553	Enlarged infected lymph nodes, pinpoint pulmonary lesions, numerous scattered abscess-like lesions in other tissues	0/6
1.0	0.25	21.4	All dead					6/6
0.25	0	20.0	20.2	22.6	24.2	740	Enlarged infected lymph nodes, pinpoint pulmonary lesions, occasional scattered abscess-like lesions throughout other tissues	0/6
0.25	0.25	20.5	20.7	18.0	All dead			6/6
0.25	0.12	20.2	20.1	20.3	20.3	360	Enlarged infected lymph nodes, extensive pulmonary lesions, few other lesions	1/6
0.25	0.25*	20.3	20.3	21.3	22.8	765	Enlarged infected lymph nodes, pinpoint pulmonary lesions, occasional scattered abscess-like lesions throughout other tissues	0/6

i.p. = intraperitoneal injection.

\*Egg yolk suspension administered intraperitoneally 48 hours after intraperitoneal injection of the bacilli.

yolk proved to be a readily reproducible phenomenon when the mixture was introduced intraperitoneally. Experiments based on the chemical fractionation of egg yolk (6) have revealed that lipovitellin and lipovitellenin, in amounts comparable to those present in it do not reproduce the effect of the whole material. However, a suspension of egg oil from the evaporated ether

extract of the whole yolk together with an alcohol-insoluble fraction of the ether extract after removal of the oil was as active as the whole egg yolk when mixed with the culture of tubercle bacilli. Furthermore, a similar effect could be obtained when egg oil was replaced by a light paraffin oil (Bayol F). Oil and the alcohol-insoluble fraction were separately ineffective. When examined microscopically, the bacilli in the mixture of culture and egg yolk suspension appeared to be located in the small globules of oil. The same finding was obtained when the culture was mixed with the suspension mixture of egg oil or paraffin oil and the alcohol-insoluble fraction of egg yolk.

TABLE V

*Response of Albino Mice of Rockefeller Institute Strain to Intraperitoneal Inoculation with H37Rv (Virulent) and H37Ra (Avirulent) Mammalian Tubercle Bacilli*

Culture	Egg yolk suspension	Weekly weight after inoculation					No. dead/ Total
		Initial	1 wk.	2 wks.	3 wks.	4 wks.	
cc.	cc.	gm.	gm.	gm.	gm.	gm.	
H37Rv 0.25	0	20.0	20.2	22.6	24.2	Killed	0/6
" 0.25	0.25	20.5	20.7	18.0	Dead		6/6
" 0.5	0	20.0	23.3	24.6	25.3	22.8	0/6
" 0.5	0.25	21.0	19.8	16.4	Dead		6/6
H37Ra 1.0	0	17.6	21.9	25.3	26.8	28.0	0/6
H37Ra 1.0	0.25	20.2	23.5	26.8	28.2	30.1	0/6
0	0	21.0	26.2	28.0	29.3	31.4	0/6

#### 4. Infection of Mice by Feeding

The following experiment was devised to test the possibility of infecting mice by feeding them once with food contaminated with tubercle bacilli cultivated in the Tween-albumin medium.

Swiss albino mice (4 weeks of age and 15 gm. in weight) and line 1 dba mice (4 weeks of age and 11 gm. in weight) were fasted for 1 day. Each mouse was then fed a piece of dry white bread (approximately 1 c.cm.) soaked immediately prior to feeding with 0.5 cc. of a 7 day old culture of H37Rv. The mice were then placed and maintained on a diet consisting of cornmeal, gelatin, butter, and a salt mixture (to be described more fully in a later publication) and sacrificed 4 weeks after the feeding with bacilli.

Autopsies revealed tuberculous pulmonary disease in all the fed animals. In this case again, the lesions were much more extensive in the dba than in the Swiss albino mice.

#### 5. Comparative Susceptibilities of Different Breeds of Mice to Experimental Tuberculous Infection

The results presented in Table I reveal a striking difference in behavior toward infection with the virulent culture H37Rv between line 1 dba and Swiss

albino mice: Whether measured in terms of minimal lethal dose of tubercle bacilli, or of rate of progression of pulmonary lesions, the susceptibility of the dba mice was much greater than that of the Swiss albino. The age (between 3 and 7 weeks) and the weight (between 12 and 20 gm.) of the animals appeared to be of little significance in determining the susceptibility to infection.

In the course of the present study, extensive comparisons have been made of the susceptibility of 22 different strains of mice obtained from commercial breeding farms or from genetical laboratories. It appears unnecessary to describe the details of these tests since their results are not entirely comparable because they could not be carried out under identical conditions.<sup>5</sup> Nevertheless, records of the results of those carried out within the last year with animals of approximately the same age (4 to 6 weeks) fed on a bread and milk diet and inoculated with cultures grown in Tween-albumin liquid medium, are sufficiently consistent to warrant the drawing of certain comparisons. In terms of minimal lethal dose of culture H37Rv and of extent of pulmonary lesions, 18 different strains of mice can be arranged approximately according to the following order of increasing susceptibility to tuberculous infection.

Mouse strain	Source from which obtained
Swiss albino	Brought by Dr. Clara Lynch to the Rockefeller Institute from Switzerland.
" "	Obtained from Dr. Lynch and raised by R. G. Hahn at the Rockefeller Institute.
" "	Viktor Schwentker, Tumblebrook Farm, Brant Lake, New York.
" "	Rockefeller Institute stock, raised by J. Pomarico.
Swiss albino (CF <sub>w</sub> )	Procured from the Rockefeller Institute stock and raised by Carworth Farms, New City, New York <sup>6</sup>
Swiss albino (CF <sub>aw</sub> )	Mutant derived from CF <sub>w</sub> . Raised by Carworth Farms.
Strong A	Procured from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and raised by J. Pomarico.
" C	" "
Buffalo	Procured from the State Institute for the Study of Malignant Diseases, 663 North Oak Street, Buffalo, New York, raised by J. Pomarico.
Rockefeller Institute strain	Heterozygous albino strain maintained at the Rockefeller Institute and to be distinguished from the so called "Swiss Albino" strains.
Chocolate	Carworth Farms
Brown spot	" "
C <sub>3</sub> H	Roscoe B. Jackson Memorial Laboratory

<sup>5</sup> It is probable that unrecognized variations in the culture, the season of the year, and other uncontrolled factors have an appreciable influence on the course and outcome of the experimental infection.

<sup>6</sup> The strains obtained from Carworth Farms were generously supplied by Mr. C. N. Wentworth Cumming.

Mouse strain	Source from which obtained
C <sub>3</sub> H	Roscoe B. Jackson Memorial Laboratory and raised by J. Pomarico.
Strong I	Procured from Dr. L. C. Strong and raised by J. Pomarico.
<i>Mus musculus domesticus</i>	Stock of Dr. Howard Schneider, Rockefeller Institute.
C57 black	Roscoe B. Jackson Memorial Laboratory—
dba line 1	“ “ “ “ “

In general, but not uniformly, pigmented animals were much more susceptible than the albino strains. A suggestion of this difference is to be found in the results of earlier experiments involving the infection of albino mice and of the C57 black strain with bovine tubercle bacilli by the intraperitoneal route (7). Difference in susceptibility was manifested in the time of survival following injection of a given amount of culture, in the minimal infective dose required to cause death within a given period of time, and in the extent and character of the pulmonary lesions. It is less certain, however, that differences in susceptibility affected the initial phases of multiplication of the injected organisms. Multiplication of the bacilli was observed to take place within a few days, even when the infective inoculum was very small, in the most resistant as well as in the most susceptible strains of mice. Similarly, intravenous injection of small amounts of bacilli resulted within 2 weeks in the production of pulmonary lesions in all animals. It was in the subsequent course of events that susceptibility and resistance became manifest. The lesions generally remained small in albino mice, with a tendency to regress when few in number. In animals of the susceptible line 1 dba and C57 black strains, on the other hand, rapid progression of the disease took place, and the presence of a few lesions was sufficient to cause the destruction of a whole pulmonary lobe within a few weeks.

The difference in susceptibility between different mouse strains is illustrated by the following experiment in which are compared three strains of mice, Swiss albino, C<sub>3</sub>H, and C57 black, which exhibit respectively high, intermediate, and low resistance to experimental tuberculous infection.

The mice used were of the following age and average weight: Swiss albino 6 weeks, 22 gm.; C<sub>3</sub>H 6 weeks, 17 gm.; C57 black 6 weeks, 17 gm. They were infected with varying amounts of H37Rv cultures grown in Tween-albumin liquid medium introduced by the intraperitoneal or intravenous route. The number of deaths occurring at weekly intervals (up to 6 weeks) is recorded in Table VI.

It is obvious that the order of increasing susceptibility of the three strains of mice, Swiss albino, C<sub>3</sub>H, and C57 black was the same, whether the infective dose was introduced intravenously or intraperitoneally, with or without egg yolk, and irrespective of whether susceptibility was measured in terms of the number of deaths occurring within a given period, or by the rapidity with which death occurred after a given infective dose. Pulmonary lesions were

TABLE VI

*Comparative Susceptibility of Three Mouse Strains to Infection with Mammalian Tubercle Bacilli via Intraperitoneal and Intravenous Routes*

Culture H37Kv	Egg yolk sus- pension	No. mice of each strain	Swiss albino						C57 black						Total dead	
			No. deaths weekly						No. deaths weekly							
			1 wk.	2 wks.	3 wks.	4 wks.	5 wks.	6 wks.	1 wk.	2 wks.	3 wks.	4 wks.	5 wks.	6 wks.		
Intraperitoneal																
cc.																
0.25	0	6						0			2	2		1		5
0.25	0.25	6						0		2	1	1		1		3
0.05	0	6						0								0
0.05	0.25	6						0			2		1	1		4
Intravenous																
cc.																
0.05	0	6						0								6
0.05	0.1	6			5			5		2	4			2	3	6
0.01	0	6						0		6				2	2	4
0.01	0.1	6						0		3	2			2	3	6

found in all infected animals, but as indicated earlier in this report, the rate of progression of these lesions was highly characteristic for each mouse strain. The lesions observed in resistant (Swiss albino) and susceptible (line 1 dba) mice are illustrated in Figs. 1 to 4. Mice of the C57 black strain gave results similar to those obtained with line 1 dba.

#### DISCUSSION

Recent studies have established that mice are more susceptible to experimental infections with mammalian tubercle bacilli than was formerly believed (7-16). Thus, intravenous injection of 0.1 to 0.2 mg. human or bovine bacilli has been found by several workers to cause death of a large percentage of albino mice within 3 to 4 weeks (8, 10, 14, 15, 16). Of special interest is the finding that intravenous inoculation with 10 to 100 living bacilli can lead to a mild chronic disease, during which the organisms proliferate although they may fail to produce visible tubercles (9). Chronic infection can also be produced by causing mice to inhale small numbers of bovine bacilli in the form of an aerosol mist (13). The results described in the present paper confirm and extend these findings. They establish in particular that one can modify almost at will the rate of progression and the outcome of experimental tuberculous infection of mice by modifying a number of factors which influence the infective organism and as will be shown later, the infected host.

It has long been known that variants of a given culture of tubercle bacillus can differ in virulence; this is illustrated in Tables II and V and will be discussed further in an accompanying communication (5). The physiological state of the culture used for inoculation is also worth consideration. Cultures grown for several weeks according to the classical techniques (on egg yolk slants, or as surface pellicles on synthetic liquid media) contain a large percentage of dead cells and of cells of differing age and physiological activity (17). It is not surprising, therefore, that these cultures have often been found ineffective in producing disease in an animal somewhat resistant to tuberculous infection, as in the albino mouse. The cultures used in the present study were young (1 week old) and homogeneous (diffuse growth); comparison of the number of cells determined by direct microscopic examination and of the number capable of initiating growth in liquid and on agar media revealed in repeated tests that most of those in our cultures were viable and physiologically active. Moreover, the diffuse character of the cultures growing in liquid Tween-albumin medium made unnecessary mechanical trituration for the preparation of homogeneous bacterial suspensions for infection. The youth and viability of Tween-albumin cultures as well as elimination of trauma in the preparation of the bacterial suspension probably accounted for its high infectivity for mice.

The mode of injection of the bacilli had a marked influence on the type of disease produced. Intravenous inoculation of the bacilli resulted in a localiza-

tion of the disease that was predominantly pulmonary; the minimal infective dose capable of causing death within a month ranged from 0.0005 to 0.02 mg. of bacilli, depending upon the strain of mice used. Introduction of the bacilli by the intraperitoneal route was much less effective and resulted in a less predictable localization of the infection. Admixture of the bacilli with egg yolk (or with a mixture of oil and the phosphatide fraction of the yolk) prior to injection markedly increased the severity of the pulmonary disease and the death rate. It is very likely, however, that this enhancement of infection was not due to an effect on the host, or on the virulence of the bacilli, but rather to some modification by the yolk material of the surface of the bacteria which caused the latter to aggregate within oil droplets. It appears probable that these small droplets containing bacilli were not phagocytized locally in the peritoneal cavity but were transported to the lung where they were deposited as infective foci. Bacilli introduced into the brain multiplied very rapidly, even when the infective dose was very small (10 to 100 bacilli); preliminary evidence indicates that in this organ bacterial proliferation takes place within the larger monocytes, at least to begin with. Surprisingly enough, the animals manifested little evidence of disease during the intracerebral phase of bacterial multiplication. Within 2 to 3 weeks after infection, however, invasion of lung tissue became manifest and the disease process took the form observed after infection by the intravenous route.

Nothing is known as yet of the factors—immunological or physiological—which hold in check the progress of the lesions in the more resistant animals. In the course of the present study, however, many observations have been made which indicate that this resistance can be decreased by a number of non-specific circumstances. Thus, as will be shown in a forthcoming publication, changes in diet can greatly shorten the survival time of mice infected with mammalian tubercle bacilli.

#### SUMMARY

Introduction of the bacilli by the intravenous route or by feeding gives rise to a disease predominantly localized in the lungs. Following intracerebral infection, the bacilli first multiply rapidly in the brain tissue, and then invade other organs, producing lesions especially in the lungs. Injection of the bacilli by the intraperitoneal route is less effective than by either the intravenous or intracerebral routes; however, admixture of the bacilli with some of the components of egg yolk increases both the infectivity and the pulmonary localization.

Different strains of mice differ markedly in their susceptibility to experimental tuberculous infection; the highest susceptibility was observed among the pigmented strains (line 1 dba and C57 black). Greater resistance does not



appear to depend on the ability to prevent the establishment of infection, but rather corresponds to a slower rate of progression of the infectious process.

It is possible to produce in mice tuberculosis presenting any desired degree of acuteness or chronicity by controlling certain factors which condition the initiation and the progression of the infection.

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## EXPLANATION OF PLATE 18

Lung sections stained with hematoxylin-eosin.  $\times 8.5$

The photographs were made by Mr. Joseph B. Haulenbeck.

FIG. 1. Mouse strain: Swiss albino.

Inoculum: 0.003 cc. H37Rv (intravenous).

Autopsy: 3 weeks after infection.

Note many small areas of parenchymal and subpleural lesions. Occasional epithelioid cells are visible in these lesions at higher magnification.

FIG. 2. Mouse strain: dba.

Inoculum: 0.003 cc. H37Rv (intravenous).

Autopsy: 3 weeks after infection.

Note large, sometimes confluent parenchymal, peribronchial, and subpleural lesions. Central necrosis is evident. Many epithelioid cells are visible in these lesions at higher magnification.

FIG. 3. Mouse strain: Swiss albino.

Inoculum: 0.01 cc. H37Rv (intravenous).

Autopsy: 3 weeks after infection.

Note several small, dark areas in the parenchyma. Higher magnification reveals these to consist of inflammatory lesions without necrosis, which are usually perivascular.

FIG. 4. Mouse strain: dba.

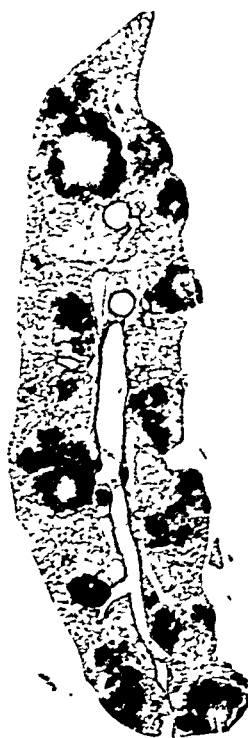
Inoculum: 0.01 cc. H37Rv (intravenous).

Autopsy: 3 weeks after infection.

Note massive consolidation of most of the lung with large areas of necrosis.



1



2



3



4



# VIRULENCE AND MORPHOLOGICAL CHARACTERISTICS OF MAMMALIAN TUBERCLE BACILLI

By GARDNER MIDDLEBROOK, M.D., RENÉ J. DUBOS, Ph.D., AND  
CYNTHIA PIERCE, Ph.D.

*(From the Laboratories of The Rockefeller Institute for Medical Research)*

PLATES 19 AND 20

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Although variations in the morphological characteristics of cultures of mammalian tubercle bacilli have long been known and extensively described, the relation of these characteristics to virulence remains uncertain. Many attempts have been made to correlate virulence and avirulence on the one hand with smoothness and roughness of colonial morphology on the other (1-5). Unfortunately, the unstable physical and chemical characteristics of the classical solid egg media have led to much confusion in terminology and description (6, 7).

The recent development of convenient techniques for the growth of tubercle bacilli in liquid and on solid media of relatively simple and reproducible composition (9-11) has permitted a reinvestigation of the morphology of these organisms. Moreover, the possibility of establishing in mice experimental tuberculous infections with small numbers of the bacilli (8) has allowed more accurate measurement and, therefore, a more thorough analysis of the relation of virulence to certain morphological characteristics of the culture.

Our study of the correlation between virulence and morphological characteristics has been based on the earlier investigations of Petroff, Steenken, and their associates (1, 12-16). By aging virulent cultures of mammalian tubercle bacilli on solid egg media, these investigators have obtained stable variant strains which are avirulent for guinea pigs. For example, the classical H37 strain has been dissociated into the highly virulent variant, H37Rv, and the stable avirulent variant, H37Ra, and certain consistently different cultural characteristics have been recognized between these two variants (17). This work has made available for comparative analysis of virulence and morphology two variant forms of the H37 strain as well as a number of other stable variants of virulent cultures of known virulence for the guinea pig.<sup>1</sup>

The present paper attempts to correlate certain differential morphological characteristics of these cultures with their virulence, measured either by the classical techniques in the guinea pig or by the recently described mouse infection tests (8).

<sup>1</sup>These cultures were obtained from the National Tuberculosis Association's Standard Culture Depot at Trudeau in the spring of 1946 and since that time have been transferred as routine in the Tween-albumin liquid medium.

*Materials and Methods*

The following strains of mammalian tubercle bacilli were used:

The two "extreme" variants of the classical H37 strain derived from a culture of a single cell: H37Rv, virulent, and H37Ra, avirulent (17).

H4Ra, reported to be avirulent for guinea pigs (14).

JH16Ra, reported to be avirulent for guinea pigs (14).

R1Rv, reported to possess low but definite virulence for normal guinea pigs and to be fatally virulent for silicotic guinea pigs, and its variant R1Ra, reported to be avirulent for guinea pigs (15).

A strain of BCG (BCG 317) obtained from the Henry Phipps Institute.

Ravenel, a classical virulent bovine strain.

Bovine 3817, a recently isolated virulent bovine strain.

Many strains recently isolated from sputum, spinal fluid, and blood of tuberculous patients.

All these strains were cultivated and transferred as routine in the previously described liquid medium containing 0.05 per cent Tween 80 and 0.2 to 0.5 per cent bovine serum albumin (fraction V) (8).

Virulence for mice was tested by intraperitoneal, intravenous, or intracerebral injection of 7 to 10 day old diffuse cultures in Tween-albumin liquid medium (8). Evaluation of virulence was based upon the ability of the culture to produce death, grossly visible lung lesions, and enlargement of the spleen, within 4 weeks after infection with various doses of living organisms.

Details concerning the preparation of basal liquid medium and its modifications have been described elsewhere (11 a).

Microscopic appearance of the cells growing submerged in liquid media was studied in the growths obtained by inoculating 0.1 cc. amounts of 6 to 10 day cultures into 5 cc. of basal medium containing various amounts of Tween 80 (0 to 0.08 per cent). Observations were also made on the morphological characteristics of the growth on the surface of liquid medium. This was prepared by adding to the basal medium 0.75 per cent glycerine (reagent or c.p. grades) before autoclaving and 0.75 per cent glucose after autoclaving (11 a). The medium was distributed in 200 cc. amounts in Blake bottles of 1 liter capacity. Each bottle was inoculated with 4 cc. of a 6 to 10 day old culture grown in basal medium containing 0.05 per cent Tween 80 and 0.3 per cent serum albumin. The Blake bottle was allowed to rest on its side for 2 days, during which time the bacteria multiplied against the glass on the bottom of the undisturbed vessel. This was then quickly tipped up and held in this position for a few seconds; much of the culture remained clinging to the side of the bottle. When the latter was returned to its horizontal position small islets of organisms were floated onto the surface of the medium and served to initiate a relatively rapid surface growth in the form of a pellicle.

For investigation of colonial morphology 0.1 cc. amounts of  $10^{-3}$  dilutions of 6 to 10 day old cultures in the routine liquid medium were inoculated onto the surface of solid media containing 1.5 per cent agar and 0.5 per cent serum albumin. Glucose, oleic acid, and Tween 80 were added as described in the text.

*Comparative Virulence of Mammalian Tubercle Bacilli for Mice and Guinea Pigs*

It has been reported in the preceding paper that the two "extreme" variants of the H37 strain differ strikingly in virulence for mice (8). Table I summarizes similar observations concerning the comparative virulence of other representative strains of tubercle bacilli. While the H37Rv, Ravenel, and Bovine 3817

strains, which are virulent for guinea pigs, initiate a rapidly progressive infection in the relatively resistant Rockefeller Institute strain of mice, the three strains, H37Ra, JH16Ra, and H4Ra, are incapable of establishing a demonstrably progressive infection in the same strain of mice. Even more striking

TABLE I  
*Comparison of the Virulence for Mice of Cultures of Mammalian Tubercle Bacilli*

Culture	Amount	Strain of mouse	Route of inoculation	No. of deaths in 4 wks.	Incidence and type of grossly visible lung lesions	Increase in size of spleen	Incidence of acid-fast rods on smear
H37Rv	cc.						
	0.5	R.I.	Intraperitoneal	0	Many, small, irregular, flat	++++	Lungs, many
	0.1	R.I.	Intravenous	All dead	Many discrete	++++	" "
	$0.1 \times 10^{-2}$	R.I.	"	0	Moderate number, very small	++	" few
	0.1	dba	"	All dead in 1 wk.	Hemorrhagic lesions	±	" many
	$0.1 \times 10^{-3}$	dba	"	0	Few large lesions	++	" "
	$0.03 \times 10^{-4}$	dba	Intracerebral	0	Few lung lesions	+	Brain, many at 7 days Lungs, many at 4 wks.
H37Ra	1.0	R.I.	Intraperitoneal	0	None	0	Lungs, none
	0.1	R.I.	Intravenous	0	"	0	" rare
	0.5	C57	Intraperitoneal	0	"	0	" none
	0.1	dba	Intravenous	0	"	+++	" rare; spleen, none
	0.03	dba	Intracerebral	0	"	0	Brain, rare at 7 days
JH16Ra	0.2	R.I.	Intravenous	0	"	0	Lungs, rare
H4Ra	0.2	R.I.	"	0	"	0	" "
R1Rv	0.1	R.I.	"	0	Scattered, small	++	" moderate
BCG 317	0.1	R.I.	"	0	" "	++	" "
Ravenel	$0.1 \times 10^{-3}$	R.I.	"	0	Moderate number, very small	++	" few
Bovine 3317	$0.1 \times 10^{-3}$	R.I.	"	0	Moderate number, small	++	" moderate

R.I. = Rockefeller Institute strain (albino)—resistant to tuberculous infection.

C57 and dba (pigmented)—susceptible to tuberculous infection.

5 or 6 mice were used in each group tested.

differences between the virulent and avirulent variants are revealed by intracerebral inoculation into the susceptible dba strain of mice. Virulent organisms in very small doses (for example,  $0.03 \times 10^{-6}$  cc. of a culture of the virulent variant of H37) are capable of early and rapid multiplication intracerebrally and cause lesions in the lungs within 4 weeks. The avirulent variant, on the contrary, in a dose 100,000 times this amount, does not appear to multiply in the brain and produces no secondary lesions. Except when the infection is overwhelmingly acute and fatal, or when it is initiated by very small doses of

bacilli, virulent variants produce marked enlargement of the spleen; this is never observed in the Rockefeller Institute strain of mice inoculated with avirulent variants.

As indicated in Table I, the R1Rv and BCG strains have been observed consistently to produce within 4 weeks small, but macroscopically visible, pulmonary lesions and some enlargement of the spleen in the relatively resistant Rockefeller Institute strain of mice infected intravenously with 0.1 cc. of undiluted culture. These two strains of mammalian tubercle bacilli possess, therefore, low but definite virulence, since they are able to multiply *in vivo*, at least for a period of time, and to produce lesions in mice. Similarly, the R1Rv strain is known to possess a low degree of virulence for the normal guinea pig (15, 16); the virulence for the guinea pig of the BCG strain used in the present studies has not been determined.

On the basis of the observations which have just been reported it appears justifiable to conclude that the virulence of mammalian tubercle bacilli, as measured by mouse infection tests, correlates well with the degrees of virulence determined by the classical techniques in the guinea pig.

### *Comparative Morphological Characteristics of Strains of Tubercle Bacilli Endowed with Different Degrees of Virulence*

1. *Comparison of H37Rv and H37Ra.*—For convenience of presentation, observations on the two "extreme" variants, virulent and avirulent, of the classical H37 strain will be reported first. Studies on other strains will be described by reference to these two variants.

Inoculation of the basal medium containing serum albumin and no more than 0.02 per cent Tween 80 with a culture (in Tween-albumin medium) recently isolated from an infected mouse gives rise within a few days to a growth consisting of bundles, ropes, or cords of strongly acid-fast bacilli in which the orientation of the long axis of each cell is parallel to the long axis of the cord. Figure 1 *b* illustrates this type of cellular arrangement. In the absence of Tween 80 or in the presence of less than 0.01 per cent of this substance, the culture appears as a maze of intertwined serpentine cords when viewed under low power magnification.

The tendency of the virulent variant to form cords can be completely inhibited by cultivation in media containing 0.05 per cent or more of Tween 80; under these conditions the culture becomes highly diffuse and consists predominantly of isolated bacterial cells. Transfer of this highly dispersed culture back to medium containing no Tween 80 or a low concentration of this substance results again in growth in the form of cords.

That cord formation is not an artifact of cultivation *in vitro* is indicated by the finding of short but definite cords and bundles of tubercle bacilli arranged in parallel in the brain tissue of mice infected intracerebrally with high dilutions



of a diffuse culture of H37Rv. Cords are also readily demonstrable in the yolk sacs of chick embryos infected with H37Rv or with other virulent cultures of mammalian tubercle bacilli.

In contrast to these morphological characteristics of the virulent variant of H37, the completely avirulent variant, H37Ra, has never been observed to form definite cords under any condition of growth. It grows, as illustrated in Fig. 1 *a*, in non-oriented clumps the size of which depends upon the age of the culture and the concentration of Tween 80 in the medium. Furthermore, the cells of H37Ra are less acid-fast, taking the methylene blue counterstain more readily than the cells of the virulent culture.

On the surface of Tween-albumin-agar media H37Rv, recently isolated from infected animals, gives rise to the type of colonies illustrated in Fig. 2 *b*. The colonies are flat and highly translucent, and display a serpentine structure in the presence of low concentrations of Tween 80. On the other hand the use of higher concentrations of Tween 80 decreases the tendency to serpentine growth on the surface of agar media as it inhibits the formation of cords in liquid media.

When inoculated onto the surface of agar media containing more than 0.005 per cent Tween 80 the avirulent variant grows in the form of the smooth, raised, opaque colonies illustrated in Fig. 2 *a*.

The most striking differences between the two variants of H37 are brought out by studying the colonial morphology on the surface of agar medium containing 0.5 per cent serum albumin and no Tween. As shown in Figs. 3 *a* and 3 *b*, these fundamental differences are independent of the size of the colony. The ability of the virulent variant to form serpentine cords which tend to spread out over the surface of the agar appears to account for all the gross morphological differences between the colonies of the two variants.

The differential morphological characteristics of the growth of the two variants of H37 on the surface of liquid medium, previously observed by Steenken (17, 18), have also been recognized in our studies. The virulent variant has a marked tendency to form a thin veil which spreads uniformly and rapidly over the entire surface of the liquid medium and climbs high on the sides of the glass container; the avirulent variant, in contrast, has much less tendency to spread and heaps up in more or less discrete islands which do not coalesce for long periods. Microscopic examination of fixed specimens of surface pellicles reveals the presence of the serpentine cords in the virulent culture and the absence of any consistent orientation in the growth of the avirulent variant. Thus the difference in macroscopic morphology on the surface of liquid medium is a reflection of the same basic difference observable microscopically in submerged culture and on solid agar media.

The morphological characteristics described above were constant over the pH range 6.0 to 7.0, and were not affected by the addition of up to 0.5 per cent glucose to the medium. Oleic acid added in a final concentration of 0.005 per

cent to albumin-agar (11 a) stimulates the growth of both variants of the H37 strain but has no effect on the fundamental differences in their morphological properties.

2. *Characteristics of Other Virulent Strains of Mammalian Tubercle Bacilli.*—All strains of tubercle bacilli freshly isolated from human pathological materials which have thus far been examined in our laboratory possess high virulence for the mouse and exhibit the morphological characteristics of the virulent H37Rv culture. Fig. 4 illustrates the typical morphology of a primary culture in Tween-albumin liquid medium of tubercle bacilli recovered from the sputum of a patient with pulmonary tuberculosis. The morphological characteristics of the virulent bovine strains, Ravenel and Bovine 3817, are also identical with those of the virulent variant of the H37 strain with respect to cord formation and strong acid fastness.













3. *Characteristics of Other Completely Avirulent Variants.*—The two variants, JH16Ra and H4Ra, which possess the same very low order of virulence as the H37Ra strain, exhibit also identical morphological characteristics with respect to their failure to form cords and their low degree of acid fastness in Tween-albumin medium. Their colonies on the surface of solid agar media are similar to those of H37Ra (see Fig. 5).

All avirulent variants thus far studied grow somewhat more slowly than do the virulent strains in the depth of Tween-albumin liquid media and on the surface of agar media. This is correlated with their slower growth on egg media (19). Moreover, it is more difficult to initiate growth of these avirulent variants in Tween-albumin medium than is the case with virulent strains, a fact particularly striking in the case of R1Ra, the completely avirulent variant of the R1Rv strain. These differences may be due to greater susceptibility of avirulent variants to the toxic effect of the unesterified oleic acid which contaminates commercial preparations of Tween 80 (20). It is worth noting, however, that once growth of an avirulent variant has been initiated in the Tween-albumin liquid medium, it is not difficult to maintain. Furthermore, the growth requirements of completely avirulent variants have not been observed by us or by previous investigators to be more exacting than those of highly virulent parent strains.

5. *Morphological Characteristics of Two Strains of Low Virulence.*—The R1Rv strain and the BCG 317 strain have been found to possess low but definite virulence for the mouse. Observations on the morphology of these strains reveal that they are more acid-fast than the completely avirulent variants but less acid-fast than highly virulent cultures grown in the Tween-albumin medium. They form cords in liquid medium in the absence of Tween 80 but not in media containing more than 0.01 per cent of this substance. Similarly, they give rise to flat, spreading colonies with serpentine structure on the surface of the albumin-agar medium containing no Tween 80; however, with 0.01 per cent, a concentration which has little if any effect on the colonial morphology of the fully virulent variants, the colonies of the R1Rv and BCG cultures are raised

and opaque like those of the completely avirulent variants in the presence of Tween 80. This effect is illustrated in Figs. 6 *a* and 6 *b* and Figs. 7 *a* and 7 *b*. Finally, BCG and R1Rv form on the surface of the liquid medium thin veils which consist of serpentine interconnected cords similar to those of the fully virulent strains.

Thus R1Rv and the BCG culture which we have studied behave as intermediates between the avirulent and fully virulent cultures of tubercle bacilli,

	No Tween	0.005% Tween	0.01% Tween	0.02% Tween
H37Rv Virulent				
BCG Low Virulent				
H37Ra Avirulent				

TEXT-FIG. 1. Representation of effect of Tween 80 on colonial morphology of typical mammalian strains.

with respect both to their virulence for the mouse and to their morphological properties.

The accompanying schema (Text-fig. 1) illustrates the observations concerning the colonial morphology of strains of widely differing virulence on the surface of the albumin-agar medium and the differential effect of Tween 80 on colonial structure.

#### DISCUSSION

The observations presented here demonstrate that the relative degrees of virulence of cultures of mammalian tubercle bacilli for mice correspond closely to the virulence of the same strains for guinea pigs. This correlation justifies

the use of the mouse for the study of the relative virulence of cultures of these microorganisms.

It has been recorded by many observers that cultures of mammalian tubercle bacilli which are virulent for the guinea pig give a spreading, veil-like, growth on the surface of liquid media whereas avirulent strains are characterized by a raised, non-spreading type of growth (1-3, 25-28). Moreover, these same observers have shown that when cultures of BCG, which gave the latter type of growth, became dissociated by any technique [resulting in cultures possessing virulence of demonstrable degree, the virulent variants exhibited spreading growth on the surface of liquid media.

That cultures of mammalian tubercle bacilli can grow in the form of microscopic serpentine cords has also been frequently observed (21-24). Our observations establish a correlation between this characteristic microscopic morphology and the virulence of the strain. It has been shown, furthermore, that the tendency of cultures of virulent tubercle bacilli to spread on the surface of liquid media and on solid media is also directly correlated with their microscopically demonstrable property of formation of cords.

It is true that certain rapidly growing non-pathogenic mycobacterial cultures can also grow as thin veils and form serpentine cords on the surface of liquid media (29, 30). However, these cultures differ in so many other respects from pathogenic strains and avirulent variants of the latter that they need not be considered here. Although it is possible that there may be discovered, in the future, avirulent cultures of slow growing mycobacteria—presumably of pathogenic origin—exhibiting the morphological characteristics of virulent strains, the following generalizations appear justified on the basis of published descriptions of cultures and of our own studies: On the one hand, all virulent strains of mammalian tubercle bacilli are strongly acid-fast and always produce microscopically demonstrable cords when grown under certain specific, readily reproducible cultural conditions. On the other hand, cultures of slow growing mycobacteria which fail to form cords under the same cultural conditions and which, therefore, grow in the form of heaped, non-spreading, pellicles on the surface of liquid media, possess in our experience and in the recorded experience of other investigators very low virulence or no demonstrable virulence. *Thus the ability of cultures of mammalian tubercle bacilli to form cords under the specific conditions of cultivation described here appears to be an essential accompaniment of virulence.* It is tempting to postulate that in analogy with available knowledge concerning other bacterial species, the gross and microscopic morphological differences between the cultures of virulent and avirulent variants of mammalian tubercle bacilli reflect specific immunochemical differences which, when identified, will assist in the understanding of the pathogenesis and immunology of tuberculous infections.

## SUMMARY AND CONCLUSIONS

Experimental infection of the mouse can be used for the determination of virulence of cultures of mammalian tubercle bacilli. The relative virulence of such cultures for the mouse is approximately the same as for the guinea pig.

Cultures of virulent and avirulent variants of mammalian tubercle bacilli grown in the depth of Tween 80-albumin liquid medium, on the surface of solid agar modifications of this medium, and on the surface of a liquid modification of this medium exhibit consistent morphological differences. All virulent cultures tend to form microscopically demonstrable serpentine cords of varying thickness and length consisting of highly acid-fast bacilli oriented in parallel along the long axis of the cord. The formation of cords appears to be an important factor in conditioning the ability of cultures to spread on the surface of liquid and solid media. It can be inhibited by the addition to the medium of the surface-active water-dispersible oleic acid ester, Tween 80. Avirulent variant bacilli grow in a more or less non-oriented fashion. They have never been observed to form cords under any condition of growth and are much less acid-fast than the virulent cultures when grown in Tween-albumin medium.

Two strains of mammalian tubercle bacilli which are intermediate in degree of virulence between the fully virulent and the avirulent variants also exhibit intermediate morphological characteristics.

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#### EXPLANATION OF PLATES

The photographs were made by Mr. Joseph B. Haulenbeek.

#### PLATE 19

FIG. 1 *a*. H37Ra. Ziehl-Neelsen stained smear of a 7 day old culture in liquid medium containing 0.02 per cent Tween 80 and 0.5 per cent serum albumin. Note the lack of orientation in the arrangement of the cells of this avirulent strain.  $\times 1000$ .

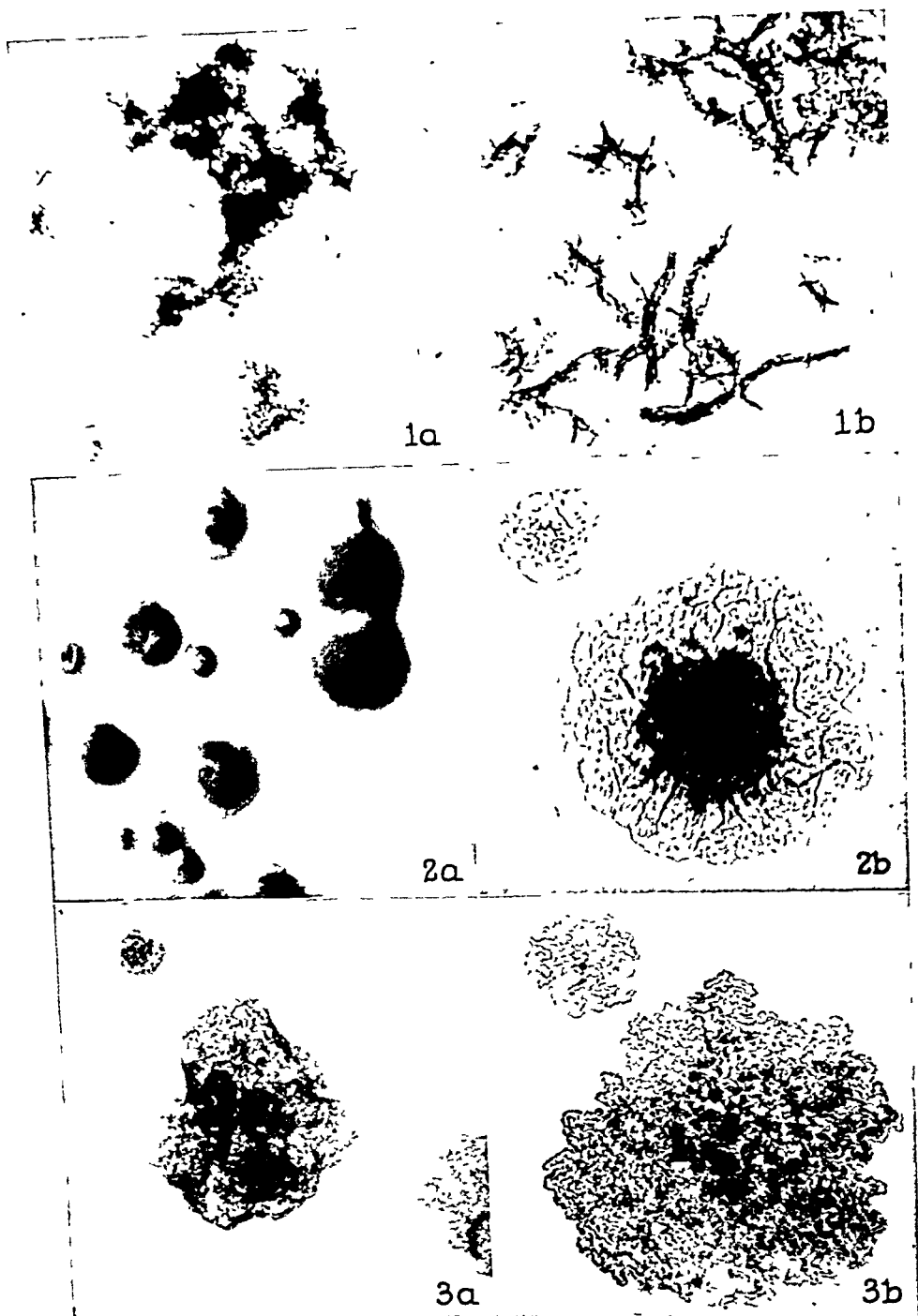
FIG. 1 *b*. H37Rv. Ziehl-Neelsen stained smear of a 7 day old culture in liquid medium containing 0.02 per cent Tween 80 and 0.5 per cent serum albumin. This culture was recently isolated from an experimentally infected mouse. Note the tendency to the formation of cords.  $\times 1000$ .

FIG. 2 *a*. H37Ra. 12 day old culture on the surface of the agar medium containing 0.01 per cent Tween 80 and 0.5 per cent serum albumin. The colonies are smooth surfaced, raised, and opaque.  $\times 90$ .

FIG. 2 *b*. H37Rv. 12 day old culture on the surface of the agar medium containing 0.01 per cent Tween 80 and 0.5 per cent serum albumin. The colonies are flat and translucent, and have serpentine markings.  $\times 90$ .

FIG. 3 *a*. H37Ra. 12 day old culture on the surface of the agar medium containing 0.5 per cent serum albumin and no Tween. Note the non-oriented structure of the colonies; the colonies are heaped-up and have little tendency to spread out over the surface of the medium.  $\times 90$ .

FIG. 3 *b*. H37Rv. 12 day old culture on the surface of the agar medium containing 0.5 per cent serum albumin and no Tween. The colonies have a serpentine structure; cords are visible in the form of loops at the thin undulate margins; and they are flat because of their tendency to spread out over the surface of the medium.  $\times 90$ .



## PLATE 20

FIG. 4. Ziehl-Neelsen stained smear of a primary culture of sputum in Tween-albumin liquid medium. The tubercle bacilli have grown in the form of intertwined serpentine cords in the depth of the liquid medium.  $\times 155$ .

FIG. 5. H4Ra. Colonies of an avirulent culture of tubercle bacilli on the agar medium containing 0.5 per cent albumin and only 0.005 per cent Tween. Even in the presence of a very small amount of Tween, smooth and almost hemispherical colonies are characteristic of avirulent variants.  $\times 125$ .

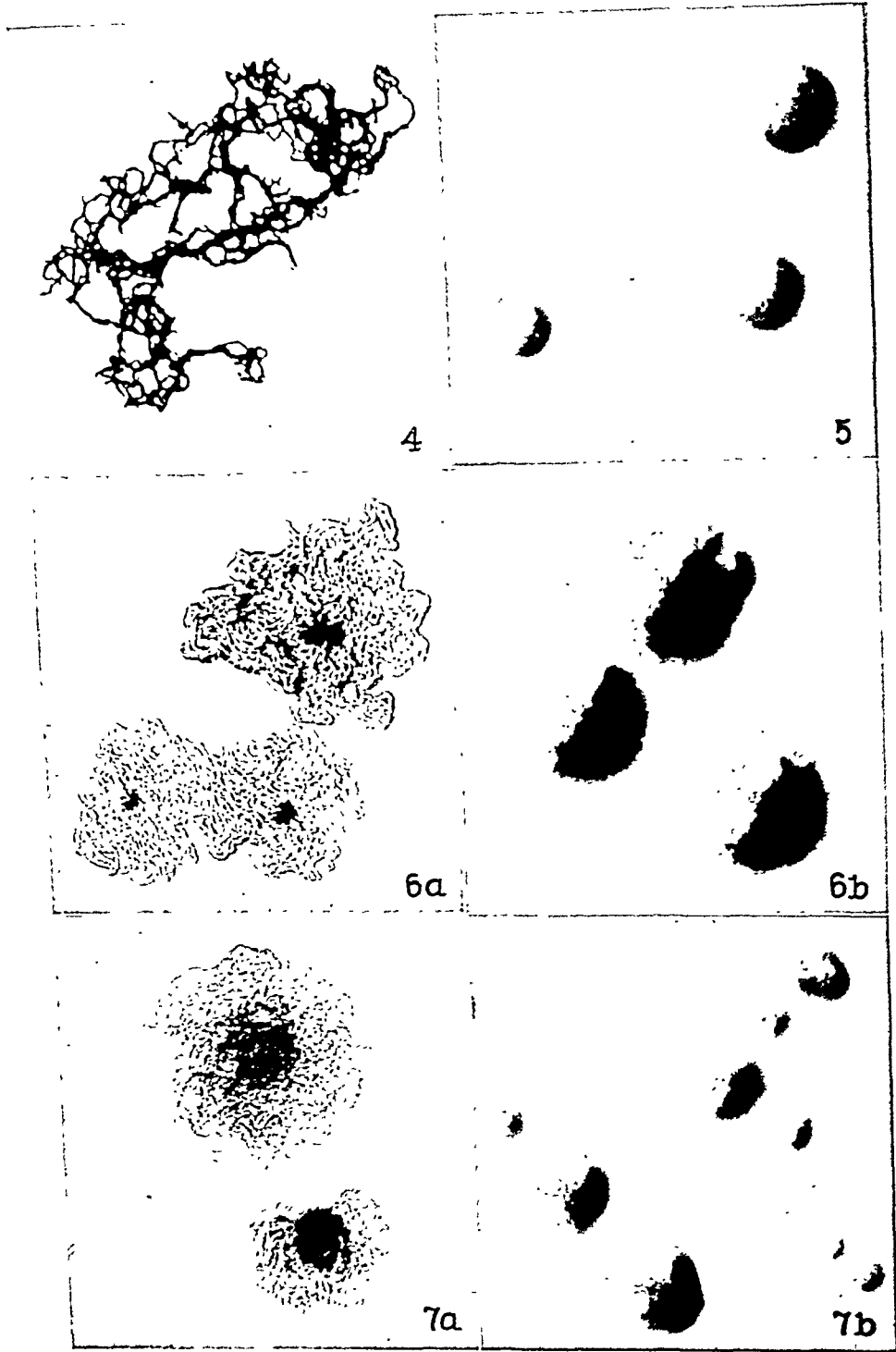
FIG. 6 *a*. R1Rv. Flat and spreading colonies of a low virulent strain on the surface of the agar medium containing 0.5 per cent albumin and no Tween. The cord structure of the colonies is visible.  $\times 125$ .

FIG. 6 *b*. R1Rv. Colonies on the surface of the agar medium containing 0.5 per cent albumin and 0.01 per cent Tween 80; for comparison with Fig. 6 *a*; the small amount of Tween has inhibited the formation of cords and the colonies are raised like those of avirulent strains growing in the presence of the same amount of Tween.  $\times 125$ .

FIG. 7 *a*. BCG 317. Colonies on the surface of the agar medium containing 0.5 per cent albumin and no Tween. The serpentine structure of the colonies is evident. Compare with the colonies of the fully virulent H37Rv culture in Fig. 3 *b*.  $\times 125$ .

FIG. 7 *b*. BCG 317. Colonies on the surface of the agar medium containing 0.5 per cent albumin and 0.01 per cent Tween. The presence of a small amount of Tween in the medium has inhibited the formation of cords by this strain. The colonies are similar to those of the avirulent H37Ra strain in the presence of the same amount of Tween (see Fig. 2 *a*).  $\times 125$ .







# CHANGES IN THE PLASMA PROTEIN PATTERN (TISELIUS ELECTROPHORETIC TECHNIC) OF PATIENTS WITH HYPERTENSION AND DOGS WITH EXPERIMENTAL RENAL HYPERTENSION

By LENA A. LEWIS, Ph.D., AND IRVINE H. PAGE, M.D.

*(From the Research Division of the Cleveland Clinic Foundation, Cleveland)*

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The modifications which occur in the plasma proteins in association with changes in the arterial blood pressure have been studied only incompletely. Variations have been reported in the plasma fibrinogen level with increases and decreases in the pressure. Goreckzy and Berencsi (1) found an increased plasma fibrinogen in 15 cases of hypertension while Gayet-Hallion, Cuny, and Quivy (2) observed lack of correlation between the level of the arterial pressure and fibrinogen concentration. Chemical methods for estimation of the fibrinogen were used. It was felt that additional information could be obtained by studying the plasma protein pattern by the electrophoresis technic and determining the relative concentration of the albumin, of  $\alpha$ -,  $\beta$ -,  $\gamma$ -globulin, and fibrinogen. The plasma of patients with essential or malignant hypertension was studied and of dogs before, during, and after the development of hypertension following wrapping of the kidneys in silk.

## *Methods and Materials*

Total plasma protein was determined by the Pregl modification of the micro-Kjeldahl method (3). Potassium oxalate was the anticoagulant used. The fractionation of the proteins was done by Longsworth's (4) modification of the Tiselius electrophoresis technic. Phosphate buffer, pH 7.8 was employed, ionic strength 0.16.

Electrophoresis studies were made of the plasma of 10 patients with essential hypertension who had no known complications likely to affect the plasma protein distribution. Studies were also made of the plasma of 16 patients with malignant hypertension, classified into 3 groups according to severity of the disease; there were 4 patients in group 1, the mildest, 8 in group 2, and 4 in group 3, the severest. The plasma proteins of 6 dogs were studied before and following the development of hypertension induced by wrapping both kidneys in silk according to the method of Page (5). Three dogs were studied to determine the effect of wrapping of another organ than the kidney, namely, the spleen.

## RESULTS

The plasma protein pattern of the 10 patients with uncomplicated essential hypertension showed as a whole only slight deviations from the normal range (Table I). Three of the 10 showed  $\alpha$ -globulin below normal both in terms of grams per 100 ml. and in percentage of total protein. Three showed slightly low  $\gamma$ -globulin in terms of grams per 100 ml., but in terms of percentage of the

total were within the normal range. The normal values obtained in this laboratory have been published in detail previously. The same conditions as employed in these studies were used (7).

In contrast with the minimal changes found in the plasma protein pattern in essential hypertension, alterations were great (Table II) in the protein pattern of patients with relatively severe malignant hypertension (groups 2, 3) (Fig. 1). The patients in group 1 showed less extensive alterations.

TABLE I  
*Plasma Protein Pattern of Patients with Essential Hypertension*

No.	Blood pressure day of protein study	Total protein	Albumin		$\alpha$ -globulin		$\beta$ -globulin		$\gamma$ -globulin		$\phi$		Mobilities, $\mu \times 10^4$ cm. <sup>2</sup> sec. <sup>-1</sup> volt <sup>-1</sup>					
													$\Lambda$	$\alpha$	$\beta$	$\phi$	$\gamma$	Dilution of plasma
	mm. Hg	gm./100 ml.	sm./100 ml.	per cent	sm./100 ml.	per cent	sm./100 ml.	per cent	sm./100 ml.	per cent	sm./100 ml.	per cent						
1	180/110	5.89	3.65	62.0	0.39	6.7	0.91	15.3	0.55	9.4	0.39	6.6	6.9	4.7	3.7	2.5	1.0	1:3
2	240/130	6.37	4.02	63.0	0.41	6.5	0.95	14.8	0.63	10.0	0.36	5.7	6.8	4.8	3.6	2.9	1.2	1:3
3	226/136	5.84	3.78	64.5	0.33	5.7	0.94	16.2	0.55	9.5	0.24	4.1						1:3
4	190/140	6.84	4.28	62.5	0.46	6.7	0.97	14.3	0.71	10.4	0.42	6.1	6.9	4.8	3.4	2.2	1.0	1:4
5	160/100	6.47	3.67	56.7	0.45	7.0	1.10	17.1	0.80	12.3	0.45	6.9	7.0	4.8	3.4	2.0	1.1	1:4
6		6.42	4.20	65.5	0.41	6.3	0.77	12.0	0.72	11.2	0.32	5.0	6.8	4.7	3.7	2.2	1.0	1:4
7		6.27	4.16	66.4	0.32	5.0	0.80	12.8	0.70	11.2	0.29	4.6	6.8	4.9	3.7	2.7	1.2	1:3
8	200/115	6.17	3.73	60.6	0.38	6.1	0.88	14.2	0.65	10.5	0.53	8.6	7.1	5.4	3.9	2.9	1.4	1:3
9		6.64	4.15	62.5	0.49	7.4	0.62	9.3	0.87	13.1	0.51	7.7	6.9	4.8	3.5	2.7	1.1	1:4
10		6.42	3.90	60.7	0.46	7.1	0.97	15.1	0.71	11.1	0.38	6.0	7.2	5.0	3.8	2.6	1.0	1:4
Normal.....		6.51	4.09	62.7	0.47	7.2	0.81	13.1	0.77	11.7	0.33	5.4						
(Average of 25, range).		5.94-7.82	3.72-5.11	60.1-67.2	0.39-0.66	6.0-8.7	0.65-1.07	11.0-15.9	0.60-0.91	8.6-14.8	0.16-0.48	2.8-7.2						

No single fraction was consistently altered, but each of the 4 in group 1 showed values outside the normal range in more than one fraction.

The changes in group 2 were much larger than in the first group. In many instances none of the protein fractions was present in normal concentration. Two of the four studies in group 3 were particularly interesting as the electrophoresis pattern showed separation of  $\alpha_1$ -globulin from the albumin. This has never been observed in normal human plasma when phosphate buffer pH 7.8 is employed, as used in these studies.

The plasma albumin was decreased in 12 patients and fibrinogen was elevated beyond the normal range in 14 of the 16 patients with malignant hypertension.

Ten of 12 patients with grade 2 and 3 malignant hypertension showed in-

creased  $\beta$ -globulin. The  $\alpha_2$ -globulin was greatly elevated in some instances while in others in the same group abnormally low values were observed. The changes observed in the plasma protein patterns of the dogs during the

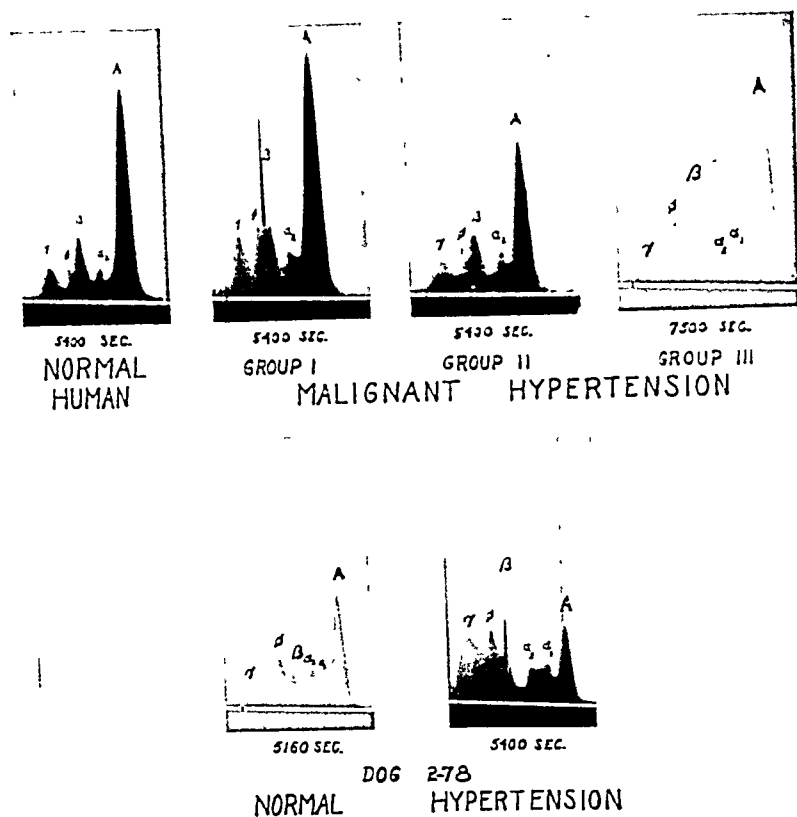


FIG. 1. Electrophoretic patterns of normal human plasma and of plasma of patients with malignant hypertension, and of normal and hypertensive dog plasma. Phosphate buffer pH 7.8, ionic strength 0.16, 0.0044 mho at 2°C. current 22 ma., time indicated under each picture.

development of hypertension are summarized in Tables III and IV. Increase in  $\gamma$ -globulin was a consistent finding. In 3 of the 6 dogs, elevation in  $\beta$ -globulin and fibrinogen level was also observed. No consistent changes in the  $\alpha_1$ - or  $\alpha_2$ -globulin occurred.

The plasma protein pattern in 2 of the 3 dogs studied whose spleens were wrapped with silk showed no significant alterations from the normal. The third dog showed some increase in  $\gamma$ -globulin and fibrinogen. This may have been due to an extensive infection of the lower jaw which developed during the period of study.

TABLE II  
Plasma Protein Pattern of Patients with Malignant Hypertension

No.	Group	Total protein		Albumin		$\alpha_2$ -globulin		$\beta$ -globulin		$\gamma$ -globulin		$\phi$		Mobilities, $\mu \times 10^5 \text{ cm.}^2 \text{ sec.}^{-1} \text{ volt}^{-1}$					Dilu- tion of plasma	
		gm./100 ml.	per cent	gm./100 ml.	per cent	gm./100 ml.	per cent	gm./100 ml.	per cent	gm./100 ml.	per cent	gm./100 ml.	per cent	A	$\alpha_1$	$\alpha_2$	$\beta$	$\phi$		$\gamma$
1	1	5.64	3.63	64.3	0.25	4.4	0.70	12.5	0.56	10.0	0.50	8.8	7.3			5.1	3.8	2.3	1.3	1:3
2	1	5.36	3.32	61.9	0.27	5.0	0.80	14.9	0.70	13.1	0.27	5.1	6.9			4.6	3.3	2.3	0.9	1:3
3	1	7.59	4.23	55.7	0.59	7.8	1.31	17.3	0.85	11.2	0.61	8.0	6.8			4.9	3.5	2.3	0.9	1:4
4	1	5.89	2.92	49.5	0.32	5.5	0.80	13.5	1.19	20.2	0.66	11.3	7.0			5.3	4.1	3.0	1.2	1:3
5	2	7.00	3.58	51.2	0.93	13.3	1.08	15.4	0.77	11.0	0.64	9.1	6.9			5.5	3.9	2.6	1.5	1:4
6	2	7.90	4.29	54.4	0.35	4.4	1.58	20.0	1.13	14.3	0.55	6.9	7.1			5.1	3.6	2.7	1.2	1:4
7	2	6.85	2.92	42.6	0.71	10.3	1.27	18.5	1.03	15.1	0.92	13.5	6.4			5.1	3.6	2.0	0.9	1:4
8	2	6.60	3.26	49.4	0.70	10.6	1.20	18.2	0.94	14.2	0.50	7.6	6.8			5.1	3.9	2.7	1.2	1:4
9	2	6.88	3.69	53.7	0.53	7.7	1.46	21.9	0.65	9.4	0.55	8.0	6.6			4.9	3.4	2.4	1.0	1:3
10	2	8.14	4.41	54.2	0.89	10.9	0.89	10.9	0.97	11.9	0.98	12.1	7.1			5.1	3.5	2.8	1.2	1:3
11	2	6.50	2.05	31.4	1.44	22.2	1.07	16.5	1.07	16.5	0.87	13.4	6.7			4.9	3.3	2.7	1.1	1:4
12	2	7.06	3.44	48.7	0.80	11.3	1.32	18.8	0.82	11.6	0.68	9.6	7.0			4.7	3.3	2.3	1.4	1:4
13	3	6.39	3.40	53.2	(0.24 gm.) $\alpha_1$ (3.7 per cent)	(0.29 gm.) $\alpha_2$ (4.5 per cent)	1.08	16.9	0.92	14.5	0.46	9.2	7.4			5.2	4.3	3.0	1.7	1:3
14	3	5.92	2.75	46.4	(0.33 gm.) $\alpha_1$ (5.5 per cent)	(0.22 gm.) $\alpha_2$ (3.8 per cent)	1.55	26.2	0.41	6.9	0.66	11.2	7.1			4.8	3.7	2.4	1.0	1:3
15	3	6.95	3.64	52.4	8.1	10.4	1.20	17.3	0.88	12.6	0.67	9.6	7.3			5.6	3.8	3.0	1.1	1:3
16	3	7.65	3.89	50.9	0.80	10.4	1.51	19.7	0.93	12.2	0.52	6.8	6.8			4.7	3.6	2.7	1.2	1:4

TABLE III  
*Plasma Protein Pattern of Dogs before and during Hypertension*

Plasma Protein Pattern of Dogs before and during hypension

Dog No.	Blood pressure mm. Hg	Total protein gm./100 ml.	Albumin		α <sub>1</sub> -globulin		α <sub>2</sub> -globulin		β-globulin		γ-globulin		ϕ	Mobilities, $\mu \times 10^4 \text{ cm.}^2 \text{ sec.}^{-1} \text{ volt}^{-1}$						
			gm./100 ml.	per cent	gm./100 ml.	per cent	gm./100 ml.	per cent	gm./100 ml.	per cent	gm./100 ml.	per cent		A	α <sub>1</sub>	α <sub>2</sub>	β	γ	ϕ	Plasma dilution
3-41	Normal	5.47	2.11	38.5	0.74	13.5	0.22	4.1	0.82	15.0	0.70	12.9	0.88	16.07	6.63	5.23	6.27	1.1	1:3	
	176	7.34	2.33	31.8	0.68	9.2	0.76	10.3	1.28	17.5	1.32	17.9	0.97	13.37	9.63	5.63	8.28	1.1	1:4	
	198	7.12	2.14	30.1	0.68	9.5	0.58	8.1	1.11	15.6	1.34	18.8	1.27	17.97	8.63	5.53	8.27	1.0	1:4	
2-78	Normal	5.03	2.08	41.2	0.47	9.4	0.63	12.6	0.54	10.7	0.54	10.7	0.77	15.47	6.64	5.43	8.28	0.9	1:3	
	210	7.28	1.73	23.7	0.73	10.0	0.29	4.0	0.98	13.5	2.18	29.9	1.38	18.97	9.65	5.43	8.27	0.9	1:3	
2-54	136(normal)	5.45	2.32	42.4	0.46	8.4	0.55	10.0	0.80	14.8	0.52	9.6	0.80	14.87	6.64	5.73	9.29	1.0	1:3	
	160	5.86	1.76	30.1	0.52	8.8	1.01	17.2	1.01	17.2	0.74	12.6	0.82	14.17	7.64	5.63	9.28	1.1	1:3	
	190	6.36	2.22	35.0	0.65	10.2	0.76	11.9	0.80	12.6	1.03	16.2	0.90	14.17	6.63	5.63	8.28	1.0	1:3	
3-58	140(normal)	4.81	2.13	44.3	0.44	9.1	0.45	9.3	0.54	11.3	0.42	8.8	0.83	17.27	6.64	5.43	9.29	0.9	1:3	
	174	5.53	1.78	32.2	0.64	11.5	0.74	13.3	0.77	13.8	1.07	19.3	0.55	9.97	6.63	5.64	13.00	0.9	1:3	
3-05	136(normal)	5.89	2.65	45.0	0.66	11.3	0.39	6.6	0.86	14.6	0.55	9.3	0.78	13.27	7.63	5.74	12.51	1.0	1:3	
	220	7.15	1.27	17.8	0.49	6.8	0.71	9.9	1.44	20.2	1.61	22.5	1.63	22.87	6.64	5.43	9.24	1.0	1:3	
4-03	140(normal)	5.76	2.36	41.0	0.55	9.6	0.67	11.6	0.96	16.6	0.55	9.6	0.67	11.67	7.64	5.53	8.30	1.1	1:3	
	170	5.72	1.93	33.6	0.69	10.9	0.91	16.1	0.77	13.4	0.84	14.7	0.58	10.27	8.62	5.34	10.29	1.0	1:3	
	230	6.51	2.18	33.5	0.52	8.0	0.63	9.7	0.84	12.9	1.41	21.7	0.92	14.27	6.62	5.43	8.29	1.1	1:3	

TABLE IV  
*Blood Pressure and Plasma Protein Studies on Dog 3-41 before, during, and following the Development of Hypertension*

Date	Arterial blood pressure mm. Hg	Total protein		Albumin		$\alpha_1$ -globulin		$\alpha_2$ -globulin		$\beta$ -globulin		$\gamma$ -globulin		$\phi$		Mobilities, $\mu \times 10^4 \text{ cm}^2 \text{ sec}^{-1} \text{ volt}^{-1}$				
		gm./100 ml.	gm./100 ml.	gm./100 ml.	per cent	gm./100 ml.	per cent	gm./100 ml.	per cent	gm./100 ml.	per cent	gm./100 ml.	per cent	gm./100 ml.	per cent	A	$\alpha_1$	$\alpha_2$	$\beta$	$\gamma$
5-28-46	Normal,*	5.47	2.11	38.5	0.74	13.5	0.22	4.1	0.82	15.0	0.70	12.9	0.88	16.0	7.6	6.3	5.2	3.6	2.7	1.1
6-6-46	pre-op																			
6-7-46	170																			
6-14-46	167																			
6-19-46	176																			
6-25-46	170																			
7-5-46	176	7.34	2.33	31.8	0.68	9.2	0.76	10.3	1.28	17.5	1.32	17.9	0.97	13.3	7.9	6.3	5.6	3.8	2.8	1.1
7-11-46	188																			
7-12-46	196																			
7-16-46	214	7.12	2.14	30.1	0.68	9.5	0.58	8.1	1.11	15.6	1.34	18.8	1.27	17.9	7.8	6.3	5.5	3.8	2.7	1.0
7-19-46	220																			
7-24-46	208	5.65	1.74	30.8	0.37	6.6	0.60	11.7	0.84	14.9	1.06	18.7	0.98	17.3	7.7	6.5	5.3	7.2	6.1	1.1
7-25-46	204																			
7-25-46	202																			

\*Normal—before operation (wrapping of kidneys with silk).



## DISCUSSION

The finding of no significant change from normal in the plasma fibrinogen in essential hypertension would indicate that no direct relationship existed between blood pressure level and fibrinogen concentration. The fact that the  $\alpha_2$ -globulin level in the patients with essential hypertension was in the lower normal range or actually decreased is interesting, because of the finding of Plentl, Page, and Davis (6) that renin substrate has the same electrophoretic mobility as  $\alpha_2$ -globulin.

Other diseases in which a low plasma  $\alpha_2$ -globulin has been observed are untreated myxedema (7) and hypoadrenal activity of pituitary origin (8). In both of these a low arterial blood pressure is present.

The finding of a greatly elevated  $\beta$ -globulin in many of the patients with malignant hypertension suggests that a modified  $\beta$ -globulin level may be an important change coexistent with vascular disease. Among the conditions in which an elevated  $\beta$ -globulin level is found are: the nephrotic phase of Bright's disease, terminal glomerular nephritis (9), diabetes (10) when uncontrolled, and in controlled patients who have diabetic retinitis and frequently also, increased capillary fragility.

The increased  $\beta$ -globulin was observed consistently in diabetic patients with progressing diabetic retinitis. In some of these cases, marked improvement in visual acuity and in the eyegrounds occurred following ingestion of a high protein diet and satisfactory diabetic control for long periods. Simultaneously, the plasma albumin level approached normal levels and in some cases the  $\beta$ -globulin decreased to normal. In the patients who failed to show improvement in the eye grounds as a result of therapy, no shift toward normal levels was observed in the plasma protein pattern.

The partial separation of  $\alpha_1$ -globulin from albumin during electrophoresis of human plasma, when phosphate buffer pH 7.8 is employed, is observed very infrequently. It has never been encountered in normal plasma. In the last 750 electrophoresis studies carried out on human plasma at the Cleveland Clinic, 14 showed this phenomenon. In the group of patients were 2 with malignant hypertension, 3 with glomerulonephritis, 3 with cirrhosis of the liver, 2 with avitaminosis, 3 with lupus erythematosus, and 1 with congestive heart failure. In all these the plasma protein pattern was markedly abnormal.

The fact that little change was observed in the plasma protein pattern of the dogs whose spleens were wrapped with silk indicates that production of scar tissue induced by foreign irritants in the abdomen is not in itself cause for the increased  $\gamma$ -globulin observed in the hypertensive animals. There was no direct correlation in all cases between the blood pressure and the  $\gamma$ -globulin level in the animals with hypertension, however. In one example (dog 3-41) the  $\gamma$ -globulin level was markedly increased when the blood pressure was only

moderately elevated and failed to rise much higher when the pressure rose from 176 mm. Hg to 198 mm. Hg. It is interesting that the one dog showing marked increase in  $\beta$ -globulin, both in grams per 100 ml. and in percentage of total protein, was the animal exhibiting the malignant syndrome as evidenced by papilledema and detachment of the retinas. Further investigation of this phase of the problem is in progress.

#### SUMMARY

The plasma protein pattern of patients with uncomplicated essential hypertension showed only slight variations from the normal while that of patients with severely malignant hypertension showed marked shifts. The fibrinogen and  $\beta$ -globulins were usually elevated beyond the normal range and the albumin decreased. In less severely malignant hypertension, the changes were less marked.

In dogs with experimental renal hypertension, the  $\gamma$ -globulin level was greatly elevated, and in one animal exhibiting the malignant syndrome  $\beta$ -globulin and fibrinogen were also increased.

Elevation of  $\beta$ -globulin seems in some manner associated with the occurrence of severe vascular disease.

We wish to thank Dr. Robert D. Taylor for cooperation in sending suitable patients for study and Mr. James Clark who rendered valuable technical assistance.

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# THE TOXIC ACTION OF PREPARATIONS CONTAINING THE OXYGEN-LABILE HEMOLYSIN OF STREPTOCOCCUS PYOGENES

## III. INDUCTION IN MICE OF TEMPORARY RESISTANCE TO THE LETHAL EFFECT OF THE TOXIN\*

BY ALAN W. BERNHEIMER, Ph.D., AND G. L. CANTONI, M.D.

(From the Department of Bacteriology, New York University College of Medicine,  
New York, and the Department of Pharmacology, Long Island College of  
Medicine, Brooklyn)

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*Streptococcus pyogenes* forms a substance which has a toxic action on the isolated frog's heart. The action is not a direct one because a single application of the preparation of toxin, although without obvious effect, causes the heart to become sensitive to a second application of the toxin (1). It has been shown that the sensitizing action of the first application is accompanied by the release from the heart tissues of a protective substance or "toxin inhibitor" (2). The toxin inhibitor is characterized by its capacity to prevent the cardiac action of the toxin (systolic contracture) and by its capacity to neutralize the lethal factor present in the streptococcal preparation employed (2). There is reason to believe that the streptococcal toxin causing these effects is similar to, if not identical with streptolysin O, the oxygen-labile hemolysin of streptococci. Proof of identity must await preparation of the material in pure form.

Consideration of the above findings raises the question of whether the injection of streptococcal toxin into animals produces an effect comparable with that observed using the isolated frog's heart, namely, the release of a toxin inhibitor. Assuming that the tissues of an animal respond to the toxin by releasing into the circulation an inhibitor, two possibilities present themselves: (1) The inhibitor remains in the circulation causing the animal to become refractory to the subsequent injection of a dose of toxin lethal for normal members of the species. (2) The release of inhibitor is followed by its removal from the blood stream, and by analogy with the isolated frog's heart, the animal would become more susceptible than normal to the lethal effect of the toxin.

It follows that the injection of a sublethal dose of toxin into mice should

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either increase or decrease their susceptibility to a second injection of toxin. Preliminary experiments designed to demonstrate decreased susceptibility were positive while experiments in which the other alternative was tested were negative. The experiments demonstrating the existence of a temporary refractory state in mice following the injection of a sublethal dose of toxin are described in this communication.

### *Methods and Materials*

The smooth variant of the C203 strain of *Streptococcus pyogenes* was employed. Concentrates of the hemolysin-containing fraction of culture supernates were prepared according to the method previously described (1) with slight modifications. A single batch of toxin (No. 783)<sup>1</sup> was employed in all the experiments, and following the practice used in previous reports it is referred to as SPA (streptococcal preparation A). Unless otherwise indicated, the SPA was activated with an equal volume of 1 per cent cysteine for 10 minutes at room temperature at pH 6.8 to 7.0. The activated SPA was diluted with sterile saline to the desired volume prior to injection into mice.

Swiss albino mice, strain CFW, weighing 16 to 20 gm., were employed. The SPA and other solutions used were diluted in sterile saline solution to a final volume of 0.4 cc. per mouse. Unless otherwise noted, all injections were intravenous.

### EXPERIMENTAL

*Mortality Rate as a Function of Dose.*—In order to observe the effect of the dose on the mortality rate, various amounts of SPA were injected into groups of mice. A total of 56 mice was used in this experiment. The results are shown in Fig. 1 from which it can be seen that the LD<sub>50</sub> is 0.015 cc. of SPA. Unless otherwise indicated, mice that died within 3 hours were counted as deaths; all others as survivals. Although all mice were observed for a period of at least 72 hours, the 3 hour death time was selected because delay in death beyond this time did not commonly occur.

*Induction of Resistance to the Lethal Effect of the Toxin.*—The effect of a sublethal dose of toxin was tested by injecting 22 mice with 0.005 cc. of SPA. Twenty-one hours later the mice received a second or "challenge" dose of 0.02 cc. of SPA. The mortality rate was 7/22 or 32 per cent. The mortality rate of 24 mice receiving 0.02 cc. of SPA, but which had not received a preliminary injection of SPA was 17/24 or 71 per cent. This experiment has been repeated on 4 occasions with similar results (Table I, part A). The average mortality rates of the 4 experiments were 29 per cent for 80 experimental mice, and 89 per cent for 62 control mice. The results show that the injection of a sublethal dose of SPA conferred a significant degree of resistance to the lethal effect of the SPA.

In the course of experiments which extended over a period of several months,

<sup>1</sup> The glutamine used in the cultivation of the streptococci was generously supplied by Lederle Laboratories, Inc.

there occurred some variation in the mortality rate produced by a constant dose of toxin. The variation was presumably due to differences in the susceptibility of different lots of mice or to slight changes in potency incurred in the preparation of the dialyzed SPA. In order to eliminate this source of error, a dose which killed 66 to 100 per cent of untreated mice was always employed as the challenge dose. The preliminary or sublethal dose, in every case was one-fourth as large as the challenge dose. It is probable that the size of the first dose is critical for the production of the refractory state. Incidental observations suggest that resistance to the toxin fails to develop when the first dose is less than  $1/4$  to  $1/6$  the challenge dose.

*The Refractory State as a Function of Time.*—Since a sublethal dose of SPA

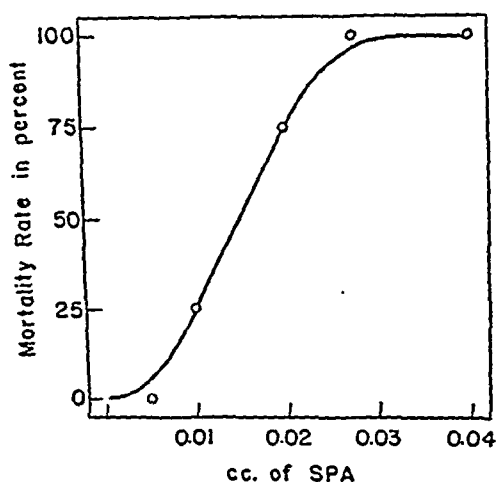


FIG. 1. Mortality rate of mice as a function of dose of SPA.

decreased the susceptibility of mice to a second injection of the same material, it was considered of interest to study the refractory state as a function of time. Groups of mice were injected with a sublethal dose, and challenged after the following intervals of time: 0.5, 3, 6, 12, 21, 28, and 40 hours. The mortality rates of these and of corresponding control groups of mice are shown in Table I, part B. The development of resistance is delineated by plotting against time the mortality rate of experimental mice divided by the mortality rate of control mice (Fig. 2). The results show that resistance sets in between 3 and 6 hours, persists until the 28th hour, and disappears by the 40th hour.

*Nature of the Refractory State and Specificity of Its Induction.*—It is evident from the time relationships that the toxin-induced protection is not due to antibody formation in the usual sense of the term. As a rule antibodies are not detectable until 40 to 60 hours after the first time an animal has been exposed

to a given antigen. It is notable that the SPA-induced protection appears in approximately 1/10 this time. Although the rapidity of the response

TABLE I

*Induction of Refractory State in Mice by Preliminary Injection of a Sublethal Dose of SPA*

Preliminary treatment	Challenge dose SPA	Time interval between doses	No. of mice injected	Mortality rate
<i>A. Demonstration of refractory state</i>				
	<i>cc.</i>	<i>hrs.</i>		<i>per cent</i>
0.005 cc. SPA.....	0.020	21	22	32
None.....	0.020	—	24	71
0.005 cc. SPA.....	0.020	20	40	23
None.....	0.020	—	12	58
0.007 cc. SPA.....	0.028	23	20	50
None.....	0.028	—	20	100
0.007 cc. SPA.....	0.028	21	20	15
None.....	0.028	—	20	100
<i>B. Induction of refractory state as a function of time</i>				
0.007 cc. SPA.....	0.028	0.5	10	90
None.....	0.028	—	10	100
0.007 cc. SPA.....	0.028	3	16	100
None.....	0.028	—	20	100
0.007 cc. SPA.....	0.028	6	20	35
None.....	0.028	—	18	100
0.007 cc. SPA.....	0.028	12	20	15
None.....	0.028	—	20	80
0.007 cc. SPA.....	0.028	28	19	21
None.....	0.028	—	15	67
0.005 cc. SPA.....	0.020	40	20	75
None.....	0.020	—	24	71
0.005 cc. SPA.....	0.020	40	12	58
None.....	0.020	—	12	67

is comparable with that of an anamnestic reaction, a specific anamnestic response would seem to be ruled out by the temporary nature of the protection as well as by failure of unactivated toxin to stimulate protection (Table II).

The work of Dougherty, Chase, and White (3) suggests that the mechanism of the non-specific anamnestic response involves the liberation of preformed

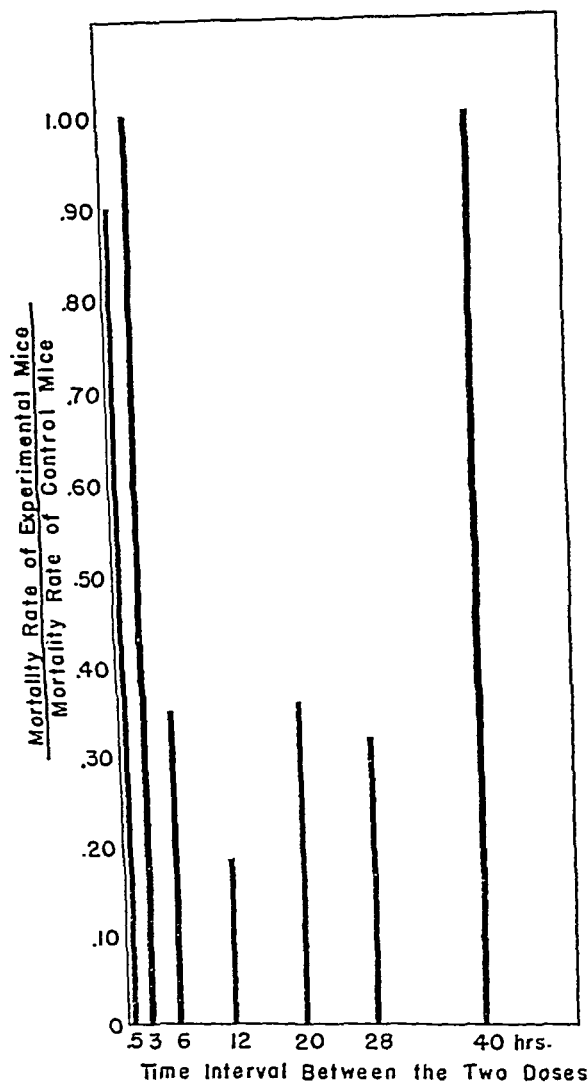


FIG. 2. Development and duration of refractory state.

antibodies from lymphocytes, the release occurring through the operation of a hormonal mechanism. The results of these investigators led us to test adrenal cortical extract and benzene for their capacity to induce refractoriness, the latter

having been shown to bring into play the necessary endocrine response. Both agents failed to induce the refractory state (Table II). The results indicate that refractoriness depends upon neither newly formed nor preformed antibody.

*Specificity of the Refractory State.*—A series of experiments were conducted in order to find out whether the resistance induced by SPA was directed only toward SPA or to other toxic agents as well. For the most part, agents were chosen which were cardiotoxic and hemolytic and therefore had pharmacological activity more or less similar to that of SPA. Since the refractory state lasts only 30 to 40 hours it was necessary to choose agents that produced death

TABLE II

*Failure of Refractory State to Appear after Injection of Unactivated SPA, Benzene, or Adrenal Cortical Extract*

Preliminary treatment	Challenge dose SPA	Time interval between doses	No. of mice injected	Mortality rate
	<i>cc.</i>	<i>hrs.</i>		<i>per cent</i>
0.007 cc. SPA*.....	0.028	23	19	84
0.007 cc. 0.5 per cent cysteine....	0.028	23	20	80
None.....	0.028	—	20	100
0.003 cc. benzene†.....	0.028	21	20	100
None.....	0.028	—	30	93
0.25 cc. adrenal cortical steroids in oil§.....	0.028	20	14	79
None.....	0.028	—	16	100
0.005 cc. SPA  .....	0.020	21	22	32
None.....	0.020	—	24	71

\* SPA not activated with cysteine.

† Injected subcutaneously; all other injections were intravenous.

§ Kindly supplied by Dr. G. Upjohn, Kalamazoo, Michigan.

|| The mortality rate of this group of mice forms part of Table I, part A.

within a few hours after injection of small doses. The dose of each agent killing between 50 and 100 per cent of normal mice was determined. This amount of each agent was injected into normal mice and into mice prepared by a preliminary injection of the standard sublethal dose of SPA. The mortality rates and survival times of the various groups of mice are shown in Table III.

Inspection of Table III reveals that SPA failed to decrease the susceptibility of mice to the lethal effect of *Cl. septicum* toxin, *Shigella paradysenteriae* endotoxin, ricin, *Bothrops schlegelii* venom, tyrocidine, and sodium taurocholate. The results with *Cl. welchii* alpha toxin were less clear cut. Although there was



TABLE III  
Specificity of Refractoriness to SPA

Preliminary treatment	Challenged with	Time interval between doses	No. of mice injected	No. of mice which survived					Mortality rate	Conclusion concerning development of refractoriness
				15 min.	15-60 min.	1-3 hrs.	4-16 hrs.	16-61 hrs.	61 hrs.	
		hrs.							per cent	
0.007 cc. SPA	0.25 mg. saponin*	20	19	0	0	0	0	2	17	11
None	0.25 mg. saponin	—	20	0	0	2	7	8	3	90
0.10 mg. saponin	0.25 mg. saponin	20	20	0	1	1	0	0	18	10
None	0.25 mg. saponin	—	20	0	0	1	12	7	0	100
0.10 mg. saponin	0.028 cc. SPA	20	20	1	4	8	2	1	4	60
None	0.028 cc. SPA	—	10	10	0	0	0	0	0	100
0.007 cc. SPA	0.003 cc. alpha toxin of <i>Clostridium welchii</i> §	20	20	0	0	1	9	6	4	80
None	0.008 cc. alpha toxin of <i>Clostridium welchii</i> §	—	20	0	0	13	6	0	1	95
0.0125 cc. SPA	0.003 cc. alpha toxin of <i>Clostridium welchii</i>	20	20	0	2	1	9	3	5	80
None	0.008 cc. alpha toxin of <i>Clostridium welchii</i>	—	20	0	0	9	8	3	0	100
0.007 cc. SPA	0.001 cc. <i>Clostridium septicum</i> toxin	20	20	0	5	14	1	0	0	100
None	0.001 cc. <i>Clostridium septicum</i> toxin	—	20	1	13	5	1	0	0	100
0.011 cc. SPA	0.4 mg. <i>Shigella paradyenteriae</i> endotoxin¶	20	20	0	0	1	14	3	2	90
None	0.4 mg. <i>Shigella paradyenteriae</i> endotoxin	—	20	0	0	1	8	1	10	50
0.007 cc. SPA	2 gamma ricin**††	15	21	0	0	0	17	4	0	100
None	2 gamma ricin††	—	20	0	0	0	16	2	2	90
0.007 cc. SPA	0.15 mg. <i>Bothrops schlegelii</i> venom§§	20	20	1	13	5	1	0	0	100
None	0.15 mg. <i>Bothrops schlegelii</i> venom	—	20	0	9	7	0	0	4	80
0.011 cc. SPA	0.25 mg. tyrocidine	20	20	1	13	2	0	0	4	80
None	0.25 mg. tyrocidine	—	20	0	18	0	0	0	2	90
0.010 cc. SPA	5.0 mg. sodium taurocholate¶¶	20	20	10	0	0	0	0	10	80
None	5.0 mg. sodium taurocholate	—	20	13	1	0	0	0	6	100

\* Commercial variety obtained from Merck and Co.

† For explanation, see text.

§ Kindly supplied by Dr. Mark H. Adams, New York.

|| Prepared according to (4).

¶ Kindly supplied by Dr. Ely Perlman, New York.

\*\* Kindly supplied by Prof. R. K. Cannon, New York.

†† Injected intraperitoneally, all other agents injected intravenously.

§§ Kindly supplied by Lic. Luis Bolaños, San José, Costa Rica.

||| Recrystallized from crude material obtained from Merck and Co.

¶¶ Obtained from Yamatichi Chemical Co.

no difference in the mortality rates of normal mice and treated mice, the latter, when challenged with alpha toxin, survived somewhat longer than normal mice. The findings indicate that the refractoriness developing in mice injected with streptococcal toxin is in general directed specifically against the homologous toxin. However, as shown in the subsequent section, cross-refractoriness occurs with saponin.

*Experiments Using Saponin.*—A curious and unexpected relationship was revealed when mice injected with a sublethal dose of SPA were later challenged with a lethal dose of saponin. Mice treated in this way exhibited a degree of refractoriness to saponin comparable to that which had been found to develop against SPA itself (Table III). It is apparent, therefore, that the protective mechanism is effective against both saponin and SPA. Experiments were then carried out to determine whether a sublethal dose of saponin confers protection against a lethal dose of saponin, and also whether resistance to SPA can be induced by the injection of a sublethal dose of saponin.

Forty mice were injected with 0.1 mg. saponin. Twenty hours later, half of these mice were challenged with 0.25 mg. saponin and half with 0.028 cc. of SPA. Only 2 of the 20 mice which had been pretreated with saponin were killed by 0.25 mg. saponin while all of 20 normal mice were killed by 0.25 mg. saponin. Of the 20 mice which were pretreated with saponin and challenged with SPA, 80 per cent died as compared with 100 per cent of normal mice. However, as Table III shows, 75 per cent of the pretreated mice survived longer than 1 hour while 100 per cent of the normal mice survived less than 15 minutes. In summary, the results show that a sublethal dose of either SPA or saponin induces refractoriness to the effect of a lethal dose of either SPA or saponin. The results of experiments in which the cardiotoxic actions of SPA and saponin were compared, form the subject of a separate communication (5).

#### DISCUSSION

Following the injection of a sublethal dose of a toxic preparation from *Streptococcus pyogenes*, there develops in mice a state of increased resistance to the lethal effect of the preparation. The increased resistance is characterized by three features: (1) The resistance manifests itself quickly (3 to 6 hours) and is of temporary duration (30 hours). (2) The degree of protection is relatively small. (3) The refractoriness exhibits a considerable degree of specificity.

The development of resistance in animals following exposure to injury has been repeatedly described in connection with investigations of the physiological and pharmacological effects of a wide variety of chemical and physical agents. Many of these observations have been reviewed in connection with what has been termed "the alarm reaction" which is said to be part of a "general adaptation syndrome" (6). The alarm reaction is defined as "the sum of all non-specific systemic phenomena elicited by sudden exposure to stimuli to which

the organism is quantitatively or qualitatively not adapted." The refractory state which we have studied is relatively specific. Whether or not it depends upon "non-specific systemic phenomena," we cannot say. So far as we are aware, resistance of the type described in this report has seldom been observed in connection with bacterial toxins.

A temporarily increased resistance of pigeons to the effect of a lethal dose of the venom of *Crotalus adamanteus* has been described by Kyes, Markin, and Graham (7). The increased resistance was induced by the injection of a sublethal dose of the venom. The similarity in time relationships and the degree of resistance described by these authors suggest that the refractoriness observed by them in pigeons and that observed by us in mice may depend upon comparable physiological processes.

The mechanism underlying the development of the refractory state is obscure. It was considered worthwhile to compare the erythrocytes of normal mice with those of refractory mice for sensitivity to the hemolytic action of SPA, particularly in view of the fact that increased resistance of erythrocytes to lysis is said (8) to follow the injection of certain hemolytic agents. No difference was found between the sensitivity to SPA of erythrocytes of normal mice and erythrocytes of refractory mice. Similarly, the antihemolytic action of plasma of normal mice was found to be no less than that of the plasma of refractory mice. As was pointed out in the introduction, the experiments with mice were undertaken in anticipation of the possibility that exposure of mice to a sublethal dose of SPA might give rise to the appearance of an SPA inhibitor in the blood stream. Although the findings can be explained by assuming that an SPA inhibitor is present in the circulating blood, experiments designed to reveal the presence of the inhibitor have been negative. It is possible that the increase in resistance reflects changes in the state of the serum lipids and that the tests thus far performed are not adequate to detect such changes. In this connection, it is notable that the toxic actions of both SPA (1) and saponin (9) are inhibited by cholesterol. There also remains the possibility that the refractoriness is not due to a humoral mechanism at all, but depends upon a lessened sensitivity of the tissues of the heart or other organs upon which the toxin acts.

The chief implications of the findings reported are: (a) there exists in the mouse an "antitoxic" mechanism which is dependent upon processes distinct from those commonly accepted as underlying immunological phenomena; (b) the mode of action of saponin and streptococcal toxin may be fundamentally similar.

It would be of interest to know whether bacterial toxins other than those investigated can also induce refractoriness, and whether other species of mammals are capable of developing resistance comparable in specificity to that shown by the mouse.

## SUMMARY

1. The susceptibility of mice to the lethal effect of preparations containing the oxygen-labile hemolysin (streptolysin O) of group A hemolytic streptococci has been studied. Injection of a single sublethal dose of the streptococcal preparation causes the development of resistance to the effect of a lethal dose injected subsequently.

2. Resistance is demonstrable 3 to 6 hours after the injection of the streptococcal preparation, persists for approximately 30 hours, and then disappears.

3. Resistance induced by the streptococcal preparation, although relatively specific, is directed not only against the streptococcal preparation but also against saponin. Mice made refractory to the streptococcal preparation and to saponin exhibit normal susceptibility to a number of other toxic agents, with the possible exception of the alpha toxin of *Cl. welchii*.

4. Mice injected with a sublethal dose of saponin develop resistance to the effect of a lethal dose of either saponin or the streptococcal preparation.

5. Resistance depends upon processes distinct from those underlying classical antitoxic immunity.

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# THE ENHANCING EFFECT OF CONCURRENT INFECTION WITH PNEUMOTROPIC VIRUSES ON PULMONARY TUBERCULOSIS IN MICE

BY MOGENS VOLKERT, M.D., CYNTHIA PIERCE, Ph.D., FRANK L.  
HORSFALL, JR., M.D., AND RENÉ J. DUBOS, Ph.D.

*(From the Hospital and the Laboratories of The Rockefeller Institute  
for Medical Research)*

PLATES 21 AND 22

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It is well established that healthy mice may harbor in their lungs one or more viruses in a latent state and it has been shown that various non-specific stimuli may serve to provoke or activate these agents with the result that infection develops (1). As a consequence, a number of experimental manipulations, especially those in which native material of animal origin foreign to the host are injected, may incur the risk of inadvertent provocation of a latent virus. Since infection of mice with mammalian tubercle bacilli can result in chronic disease, it appeared possible that the course of tuberculous infection in these animals might be influenced by the concurrent development of infection with a latent virus. It would be important to know, therefore, whether viral infection of the lungs of mice, if superimposed upon infection with tubercle bacilli, can alter the course and outcome of the infection caused by the bacterium alone.

It is the purpose of this paper to present the results of experiments in which mice given tubercle bacilli intraperitoneally were also given small amounts of either pneumonia virus of mice (PVM) or influenza A virus (PR8) intranasally. It will be shown that, following infection with either of these pneumotropic viruses, tuberculous lesions in the lungs of mice develop more rapidly and become more extensive than in control animals infected only with tubercle bacilli.

## *Methods*

*Tubercle Bacillus.*—One mammalian strain of tubercle bacillus, H37RV, was used. It was maintained in liquid culture in Tween-albumin medium and frequently was passed through mice (2, 3). The virulence for mice of H37RV maintained in this manner has been found to be constant (3). Throughout this study 7-day-old cultures in Tween-albumin liquid medium were employed; they contained approximately  $10^8$  to  $10^9$  living microorganisms per cc. As routine, the bacteria were injected by the intraperitoneal route in a total volume of 0.20 to 0.25 cc. Undiluted culture or culture diluted to varying degrees in sterile Tween-albumin medium was employed as indicated in the text. The quantity of tubercle bacilli injected is indicated in the tables in terms of the amount of culture present in the inoculum.

*Viruses.*—Pneumonia virus of mice (PVM), strain 15 (4), and the PR8 strain (5) of influenza A virus were employed. Each virus was maintained by occasional lung passage in Swiss mice and suspensions of infected mouse lungs were stored at  $-70^{\circ}\text{C}$ . As routine, the desired virus was diluted in broth containing 10 per cent normal horse serum and given by the intranasal route in a volume of 0.05 cc. to each mouse under light ether anesthesia. The quantity of virus inoculated was calculated from the 50 per cent maximum score end point (*i.e.*, M.S.50) (6) which, prior to the experiment, was determined in mice of the strain employed. In certain experiments an amount of virus equal to 1 M.S.50 dose was used; in others, still smaller amounts were employed and they are indicated in the tables as fractional parts of 1 M.S.50.

*Mice.*—Two strains of albino mice were used. One, the so called Rockefeller Institute strain, was employed in Experiments 1 and 2; the other, a strain of Swiss mice obtained from a commercial breeder,<sup>1</sup> was employed in Experiments 3 to 7. The age of mice at the time of inoculation was 4 to 5 weeks. During the experimental period the mice were kept in glass jars, 5 to 6 animals in each, and were fed a diet of white bread and milk. It is important to emphasize that the composition of the diet has a marked influence on the course and outcome of tuberculous infection (7). The susceptibility of Rockefeller Institute and Swiss strains of mice to infection with tubercle bacilli, H37RV, was similar. The two strains of mice showed, however, different susceptibilities to infection with PVM. The M.S.50 end point with PVM was, therefore, determined by separate titrations in each strain of mice and the amount of virus given was calculated from the results obtained in mice of the same strain. In Experiments 1 to 3 an observation period of 3 weeks was employed; in Experiments 4 to 7 the observation period was extended to 6 weeks.

*Pulmonary Lesions.*—Following an observation period of desired duration, all surviving mice were killed and their lungs examined for the presence of tuberculous lesions. Mice which died within 2 weeks following inoculation with either virus were discarded. Mice which died later than 2 weeks after inoculation and showed extensive tuberculous lesions in their lungs were included in the experimental results. The lungs from numerous mice in both the experimental and control groups were fixed, sectioned, and studied under the microscope. Pulmonary lesions induced either by PVM or PR8 are very different from those caused by tubercle bacilli and the latter can be distinguished readily on gross examination. In the present study interest centered entirely on the tuberculous lesions in the lungs of mice. To facilitate the presentation of results and to express the extent of the tuberculous lesions observed, the following notation was adopted: 0 = lungs which showed no gross tuberculous lesions;  $\pm$  = lungs which showed small reddish spots or tiny hemorrhagic areas which could not definitely be considered to be of tuberculous origin on gross examination; + = lungs which showed one or two readily visible tuberculous lesions; ++ to +++++ = lungs which showed in increasing number and extent distinct tuberculous lesions ranging from large circumscribed lesions to innumerable smaller lesions which filled almost the entire lung field; D++++ = lungs of mice which died with extensive tuberculous lesions more than 2 weeks after inoculation. For the purpose of this study all lungs which showed tuberculous lesions that were assigned a value of + or more were included as positive in the analysis of the results.

To express in a single figure all of the results obtained in a group of mice the lung lesion score ratio was determined in a manner analogous to that employed previously for lesions induced with pneumotropic viruses (6). For this purpose results ranging from + to D++++ were given corresponding numerical values from 1 to 5. The sum of the numerical scores obtained for a group of animals was divided by the maximum possible score (*i.e.*, the

<sup>1</sup> Mr. Victor Schwentker, Tumblebrook Farm, Brant Lake, New York.

number of mice in the group, multiplied by 5). The result is given in the tables as the lung lesion score ratio. This ratio possesses the advantage that it expresses not only the frequency but also the extent of tuberculous pulmonary lesions observed in a given group of animals. For example, in a group of mice which showed no lesions the score ratio would be 0.0, whereas in a group all of which died with ++++ lesions, the score ratio would be 1.0.

#### EXPERIMENTAL

*Simultaneous Infection with Tubercle Bacilli and PVM.*—Experiments were carried out to determine whether the course of tuberculous infection in mice would be altered by a superimposed infection with PVM when the bacterium and the virus were given almost simultaneously. Pierce, Dubos, and Middlebrook (3) showed previously that albino mice infected intraperitoneally with 0.20 to 0.25 cc. of undiluted culture of tubercle bacilli in Tween-albumin medium showed very few pulmonary lesions during an observation period of 3 weeks. However, extensive pulmonary lesions and often death of the mice resulted from the injection of the same quantity of tubercle bacilli when egg yolk was added to the inoculum.

Three different experiments (Nos. 1, 2, and 3) were carried out during a period of 4 months. In Experiments 1 and 2 the Rockefeller Institute strain of mice was employed; in Experiment 3 Swiss mice were employed. Each mouse was infected intraperitoneally with 0.20 or 0.25 cc. of undiluted culture of tubercle bacilli; in addition, half of the mice in each experiment were inoculated intranasally with PVM. Virus and bacteria were given on the same day. The amount of virus given was equivalent to 1 M.S.50 dose, an amount which is sufficient to cause, on the average, consolidation of approximately 50 per cent of the lung in each mouse. The observation period was 3 weeks. At the end of that time all surviving mice were killed and their lungs examined for the presence of gross tuberculous lesions.

The results are shown in Table I. It was found that in mice which received tubercle bacilli alone, 17 per cent showed pulmonary lesions (score ratio = 0.061) clearly demonstrable in the gross, whereas in mice which received tubercle bacilli and PVM (1 M.S.50) 73 per cent showed definite tuberculous lung lesions with a score ratio of 0.329. These findings indicate clearly that, under the experimental conditions employed, a superimposed infection with PVM led to a striking increase in pulmonary lesions associated with tubercle bacilli. It should be emphasized that the chief effect of concomitant infection with PVM appeared to be in accelerating the rate of the tuberculous infection in the lung. If mice in similarly infected control groups were observed for 6 weeks, the percentage which showed gross tuberculous pulmonary lesions was considerably increased.

The amount of PVM given in the experiments described above led to the development of relatively extensive viral pneumonia. It was of interest to determine whether amounts of virus sufficiently small to induce little or no pneumonia also would show a similar effect on the tuberculous process in the lungs.

Experiments were carried out both in Rockefeller Institute and Swiss strains of mice. The cultures of tubercle bacilli and the amounts given intraperitoneally were identical to those described above. The experiments (*i.e.*, 2a, 3a, and 3b) were carried out simultaneously with Experiments 2 and 3. One group of mice were given, simultaneously with the injection of tubercle bacilli, an amount of PVM equivalent to 0.1 M.S.50 dose; two other groups were given an amount of PVM equivalent to 0.01 M.S.50 dose. 0.1 M.S.50 dose of PVM induces

TABLE I

*The Effect of Simultaneous Inoculation with Tubercle Bacilli and Either PVM or PR8 on the Development of Tuberculous Pulmonary Lesions in Mice*

Ex- per- iment No.	Amount of tubercle bacillus culture I.P.*	Intranasal inoculation 0.05 cc.	No. of mice	No. of mice with indicated pulmonary lesions 3 weeks after infection with tubercle bacilli							Per cent of mice with tuber- culous pulmonary lesions, + or greater	Lesion score ratio
				0	±	+	++	+++	++++	D++++		
	cc.											
1	0.25	0	8		7				1			
2	0.20	0	15	4	8	2	1					
3	0.25	0	29	20	4	3	1	1				
Total (1, 2, 3) .....			52	24	19	5	2	1	1		17	0.061
1	0.25	PVM 1 M.S.50	11	1		3	1	6				
2	0.20	" "	12	3	1	4	3	1				
3	0.25	" "	25	8		2	8	4	2	1		
Total (1, 2, 3) .....			48	12	1	9	12	11	2	1	73	0.329
3a	0.25	PVM 0.1 M.S.50	23	3	4	2	5	3	3	3	69	0.417
2a	0.20	PVM 0.01 M.S.50	13	8		2	2	1				
3b	0.25	" "	24	10	3	3	3	4		1		
Total (2a, 3b) .....			37	18	3	5	5	5		1	43	0.189
2b	0.20	Horse serum broth	15	9	2	4					27	0.053
3c	0.25	Human serum	12	1	1	4	4	1		1	83	0.334
3d	0.25	PR8 1 M.S.50	22	12			4	4		2	45	0.273

\* In the tables I.P. = intraperitoneal inoculation.

only small lesions in the lungs of mice but is capable of causing some evidence of pneumonia in the large majority of inoculated animals. 0.01 M.S.50 dose of PVM induces very tiny lesions and, moreover, only a small percentage of inoculated animals show any evidence of pneumonia (8). The observation period in these experiments was 3 weeks. Animals which survived were then killed and their lungs examined as described above.

The results are summarized in Table I. It will be seen that 69 per cent of mice which received 0.1 M.S.50 dose of PVM showed gross tuberculous pulmonary lesions (score ratio = 0.417) and that 43 per cent of mice which received 0.01 M.S.50 dose of PVM showed similar lesions with a score ratio of 0.189. When these results are compared with those obtained in mice which



were given tubercle bacilli but no virus, it appears evident that concomitant infection with very small amounts of PVM led to a definite increase in tuberculous lesions in the lungs. Comparison with the results obtained in mice which were given 1 M.S.50 dose of virus shows that a tenfold decrease in the amount of virus inoculated did not decrease the effects of the viral infection on the course of the tuberculous infection. A decrease of 100-fold in the amount of virus inoculated caused some decrease in the effect but even this very small amount of virus was capable of causing a significant increase in the incidence of tuberculous lesions in the lung.

*The Effect of Intranasal Instillation of Serum.*—The virus employed in the experiments described above was diluted in broth containing normal horse serum which had been heated at 56°C. for 30 minutes. To determine whether intranasal inoculation with this diluent itself had any effect on the tuberculous process, a further experiment was carried out.

In Experiment 2b mice received 0.2 cc. of culture of tubercle bacilli intraperitoneally and also were given, by the intranasal route, 0.05 cc. of 10 per cent heated normal horse serum in broth. The animals were killed 3 weeks later and their lungs examined.

The results are shown in Table I. It was found that 27 per cent of the animals showed gross tuberculous pulmonary lesions with a score ratio of 0.053, a result which is not significantly different from that obtained in control animals given tubercle bacilli alone and no further inoculation.

In addition, the effect of the intranasal instillation of fresh human serum was determined. This was done because recent work in this laboratory has shown that fresh human serum is effective as a stimulus for the provocation of PVM latent in mouse lungs.

Simultaneously with the intraperitoneal injection of 0.25 cc. of undiluted culture of tubercle bacilli, mice in Experiment 3c were given intranasally 0.05 cc. of fresh human serum diluted 1:2 in saline. They were observed for 3 weeks, following which their lungs were examined.

The results are presented in Table I. It will be noted that 83 per cent of mice showed gross tuberculous pulmonary lesions with a score ratio of 0.334. Because of the length of the observation period and the fact that PVM is not demonstrable in mouse lungs later than 12 days after infection (9), it was not possible to prove directly in this experiment that latent PVM actually had been provoked in the mice infected with tubercle bacilli. There is, however, little reason to doubt that this occurred inasmuch as it was shown that the serum used was effective as a provoking stimulus for latent PVM in the strain of Swiss mice employed in the experiment. It seems probable, therefore, that the activation of PVM latent in the mouse lung results in an effect upon the tuberculous process which is similar to that induced by intranasal inoculation of the virus.

*The Effect of Intranasal Inoculation with Influenza A Virus.*—Because concomitant infection with PVM alters the course of pulmonary infection with tubercle bacilli in mice, it became of interest to determine whether infection with a different pneumotropic virus would cause a similar effect. For these experiments the PR8 strain of influenza A virus was employed.

In Experiment 3d mice were given 0.25 cc. intraperitoneally of undiluted culture of tubercle bacilli. In addition, one group of mice received intranasally an amount of PR8 equivalent to 1 M.S.50 dose. The observation period was 3 weeks.

The results are shown in Table I. It will be observed that 45 per cent of the mice which received both PR8 and tubercle bacilli showed gross tuberculous pulmonary lesions with a score ratio of 0.273. This result indicated that a superimposed infection with PR8 was capable of enhancing pulmonary tuberculosis in mice in a manner analogous to infection induced with PVM.

*Infection with Tubercle Bacilli Followed by Infection with Either PVM or PR8.*—In the experiments described above the inoculations were almost simultaneous whenever both tubercle bacilli and a pneumotropic virus were given. Further experiments were performed to determine whether infection with either of the viruses would influence the tuberculous pulmonary lesions even when initiated several weeks after injection of tubercle bacilli.

As has been shown, when 0.20 or 0.25 cc. of undiluted culture of tubercle bacilli was given intraperitoneally, a small percentage of mice developed gross pulmonary lesions during a period of 3 weeks. However, when animals infected in this manner were held for 6 weeks, the incidence of gross tuberculous lesions was increased considerably. In the present experiments it appeared desirable to extend the observation period to 6 weeks and, as a consequence, it was necessary to reduce markedly the quantity of tubercle bacilli injected. Preliminary experiments indicated that with the strain of mice used an inoculum equivalent to 0.005 cc. or less of culture would be suitable for the purpose of this study.

Two experiments (Nos. 4 and 5) were carried out in Swiss mice during a period of 5 months. In both experiments all mice were given 0.005 cc. of culture of tubercle bacilli intraperitoneally. Three weeks later the animals were divided into groups of approximately equal size. One group was given intranasally an amount of PR8 equivalent to 1 M.S.50 dose, another group was given intranasally an amount of PVM equivalent to 0.1 M.S.50 dose, and two groups received no second inoculation. Three weeks after inoculation with either virus, *i.e.* 6 weeks after inoculation with tubercle bacilli, the mice were killed and their lungs examined as in previous experiments.

The results are shown in Table II. It will be seen that among control mice given tubercle bacilli but not inoculated with either virus, 22 per cent showed gross tuberculous pulmonary lesions with a score ratio of 0.094. Among mice which received PR8 3 weeks after inoculation with tubercle bacilli, 61

per cent showed similar pulmonary lesions (score ratio = 0.238), whereas among mice given PVM under analogous conditions, 73 per cent showed gross pulmonary tuberculosis (score ratio = 0.572).

TABLE II

*The Effect of Inoculation with Either PVM or PR8 Virus Following Infection with Tubercle Bacilli on the Development of Tuberculous Pulmonary Lesions in Mice*

Ex- per- iment No.	Amount of tubercle bacillus culture I.P.	In- terval be- tween inoc- ula- tions	Intranasal inoculation 0.05 cc.	No. of mice	No. of mice with indicated pulmonary lesions 6 weeks after infection with tubercle bacilli								Per cent of mice with tuber- culous pulmonary lesions, + or greater	Les- ion score ratio
					0	±	+	++	+++	++++	D++++			
	cc.	wks.												
4	0.005		0	30	5	22	3							
5	0.005		0	25	3	13	2	4	1		2			
Total (4, 5).....					55	8	35	5	4	1	2	22	0.094	
4	0.005	3	PR8 1 M.S.50	26	1	9	5	7	4			61	0.233	
5	0.005	3	PVM 0.1 M.S.50	22	2	4	1	3	1	2	9	73	0.572	
Total (4, 5).....					48	3	13	6	10	5	2	67	0.392	
6	0.000,5		0	23	4	15	2	1			1	17	0.078	
6a	0.000,5	3	PR8 1 M.S.50	18	1	7	3	2	1	2	2	55	0.311	
6b	0.000,5	3	PVM 0.1 M.S.50	18	1	5	4	3	2		3	67	0.345	
Total (6a, 6b).....					36	2	12	7	5	3	5	61	0.323	
7	0.000,05		0	16	5	9	1				1	12	0.067	
7a	0.000,05	3	PR8 1 M.S.50	24		20	2				2	17	0.120	
7b	0.000,05	3	PVM 0.1 M.S.50	16		13	2	1				19	0.062	
Total (7a, 7b).....					40		33	4	1		2	17	0.080	

Further experiments were carried out with even smaller amounts of tubercle bacilli. In Experiments 6 and 7 Swiss mice were given intraperitoneally 0.000,5 and 0.000,05 cc., respectively, of culture of tubercle bacilli. Three weeks later the animals in each experiment were divided into three groups. One group served as controls and did not receive a second inoculation. Another group was inoculated intranasally with PR8 equivalent to 1 M.S.50 dose, and the last group received PVM in a dose of 0.1 M.S.50. Three weeks after the virus inoculation, i.e. 6 weeks after inoculation with tubercle bacilli, all mice were killed and their lungs examined.

The results are shown also in Table II. It was found that among control mice which received 0.000,5 or 0.000,05 cc. of culture of tubercle bacilli alone, 17 and 12 per cent, respectively, (score ratios = 0.078 and 0.067) showed gross tuberculous pulmonary lesions at the end of the 6 weeks observation

period. Among animals given 0.000,5 cc. of tubercle bacilli and PR8 or PVM 3 weeks later, 55 and 67 per cent, respectively (score ratios = 0.311 and 0.345) showed similar lesions. However, among mice which received only 0.000,05 cc. of tubercle bacilli and were inoculated with either pneumotropic virus 3 weeks later, the incidence of tuberculous pulmonary lesions was not significantly different from that found in control animals. These results indicate that pulmonary infection with either PVM or PR8, when superimposed upon tuberculous infection previously initiated by relatively small inocula, increases both the incidence and the extent of gross tuberculous lesions in the lungs of mice. When, however, the inoculum of tubercle bacilli was sufficiently small (*i.e.*, 0.000,05 cc.), neither virus caused a definite alteration in the course of the tuberculous process during the relatively short observation period employed.

*Histology of Pulmonary Lesions.*—Pierce, Dubos, and Middlebrook (3) have shown that the intraperitoneal inoculation of mice with a sufficient amount of tubercle bacilli causes pulmonary lesions, enlargement of the spleen, and of the abdominal and thoracic lymph nodes. In the present study, as was stated earlier, it was the tuberculous pulmonary lesions which were of chief interest.

Lungs obtained from mice given tubercle bacilli alone, and from animals also inoculated with either PVM or PR8, were studied histologically. Lungs which showed, in the gross, lesions of varying extent were selected from different groups of mice in several experiments. They were fixed promptly either in Zenker's fluid or in formalin. Numerous sections were prepared for microscopic examination; some were stained with hematoxylin and eosin and other parallel sections were stained by the Ziehl-Neelsen technique.

Microscopic examination showed that all mice given tubercle bacilli intraperitoneally developed evidence of some tuberculous infection of the lungs. This was found to be the case irrespective of the amount of tubercle bacilli inoculated. No evidence of significant qualitative differences in the tuberculous lesions was found in the lungs of mice which received tubercle bacilli and either PVM or PR8 as compared with those which received tubercle bacilli alone. However, in agreement with the gross findings, striking quantitative differences were observed. The lungs of animals infected both with tubercle bacilli and a pneumotropic virus showed, in general, more numerous and more extensive tuberculous lesions than did control animals infected only with tubercle bacilli. It was found that the histological findings in different lungs, which on gross examination had been assigned similar lesion scores (*i.e.*, from 0 to ++++) according to the notation described above, corresponded reasonably well one with another. Moreover, it appeared that the extent of the lesions visible in the gross reflected fairly closely the degree of pathological alteration demonstrable on microscopic examination.

In lungs which showed no gross tuberculous lesions (*i.e.*, = 0) only occasional submiliary tubercles were seen. These lesions were composed largely of

epithelioid cells and were situated interstitially, usually in perivascular or peribronchial areas. Infiltration with lymphocytes and plasma cells was minimal. (Fig. 1.) The number of tubercles present varied somewhat but usually 2 to 6 were found in a section of such a lung. In lungs which showed  $\pm$  lesions in the gross, the microscopic picture was but little different. The tubercles were a little larger and usually infiltration with lymphocytes was more pronounced. In lungs which showed + to ++++ lesions on gross examination, definite miliary tubercles were present. These were usually composed of a number of small confluent tubercles which were often situated in peribronchial or perivascular areas. Epithelioid cells were predominant and there was some infiltration with lymphocytes and a few plasma cells. (Fig. 2.) At the periphery of such lesions and in the adjacent lung tissue a caseous pneumonic process was often present. The alveoli in these areas were filled with exudate which contained desquamated and degenerating cells. If the tubercle was large, true necrosis was found at its center. (Fig. 3.) One or more bronchi were often invaded by the process. In such instances tuberculous bronchitis was present and the bronchial lumen was filled with exudate, desquamated and necrotic cells. When tubercles were present near the surface of the lung, the pleura was invaded frequently and localized pleuritis occurred.

In sections stained to show acid-fast microorganisms no tubercle bacilli could be demonstrated in the small submiliary tubercles. However, in the large tubercles, when necrosis was present, tubercle bacilli were visualized and sometimes were present in tremendous numbers. (Fig. 4.) Cultures of the lungs were made in certain instances and tubercle bacilli were obtained regularly even from lungs which showed no gross lesions. It should be mentioned that in no instance was it possible to find typical multinuclear giant cells in the tuberculous lesions. The histological findings demonstrated clearly that the lesions observed on gross examination of the lungs were tuberculous in nature. (Figs. 5 and 6.)

#### DISCUSSION

The evidence obtained in this study indicates that the course of experimental infection with tubercle bacilli in mice is altered by concurrent or superimposed infection with either of two pneumotropic viruses. The chief effect of the viral infections appears to be an acceleration of the tuberculous pulmonary process. In control animals which were given tubercle bacilli but were not inoculated with either virus, gross evidence of pulmonary tuberculosis developed in a small but appreciable proportion of instances. If mice were inoculated with very small amounts of a pneumotropic virus (*i.e.*, PVM or PR8) in addition to the injection of tubercle bacilli, the frequency of the occurrence of gross pulmonary tuberculosis was increased strikingly.

Microscopic study of the lesions present in the lungs confirmed the gross observations. In all lungs examined some evidence of an active tuberculous infection was found. The differences observed were quantitative, not qualitative, in character; in the lungs of control animals, as a rule, only a few small tubercles were present, whereas in the lungs of animals with a superimposed viral infection there were, in general, more and larger tubercles.

It appears of considerable interest that infection with viruses, as different one from another as are PVM and PR8, should have similar accelerating effects upon the course of pulmonary tuberculosis in mice. Not only are PVM and PR8 wholly unrelated immunologically but also they are of very different size; present evidence indicates that the dimensions of the virus particles of PR8 are of the order of 2.5 times greater than those of PVM (10, 11). Moreover, the rates of multiplication of the two viruses in the mouse lung are dissimilar; with PR8 multiplication is very rapid, and maximal titers usually are reached within 24 hours or less of inoculation (12); with PVM the multiplication rate is slow, and maximal titers usually are not reached until 6 days or more after inoculation (9). However, the pathological alterations which develop in the lungs of mice infected with PVM or PR8 are in many respects closely similar, and it is very doubtful whether lesions induced by one virus can be differentiated from those induced by the other on the basis of histological findings.

It seems probable that the observed effects on the course of pulmonary tuberculosis in mice of superimposed infection with either of the pneumotopic viruses studied are to be attributed to alterations induced in the lung as a result of viral activity and not to any hypothetical action on the tubercle bacillus itself. Even when extremely small quantities of PVM (*i.e.*, 0.01 M.S.50 dose) are given, it can be shown that infection with and multiplication of the virus occur despite the fact that no demonstrable pulmonary lesions or only occasional very minute lesions develop (4). Thus it appears that such small virus inocula may lead to the development of alterations in the normal physiology of the lung and that one expression of the abnormal status so induced may be a relatively increased susceptibility, or decreased resistance, to local invasion by tubercle bacilli. The available evidence suggests that tubercle bacilli find, in the lung infected with small amounts of either PVM or PR8, conditions which are more favorable for growth and for the rapid production of gross pulmonary lesions than are present in the normal lung.

The results obtained indicate that the tuberculous process in the mouse lung can be augmented significantly when a minimal viral infection is initiated even as late as 3 weeks after the injection of relatively small amounts of tubercle bacilli. In this manner it is possible, by experimental means, to convert a mild and but slowly progressing tuberculous infection, which would lead to the development of gross pulmonary lesions in only a small proportion of animals

under the conditions employed, into a distinctly more serious and rapidly progressive disease which results in the appearance of gross pulmonary lesions in a large proportion of animals.

#### SUMMARY

The course of pulmonary tuberculosis in the mouse appears to be accelerated as a result of concurrent infection of the lung with either of two pneumotropic viruses. This effect is obtained with virus inocula sufficiently small as to induce little or no definite viral pneumonia.

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## EXPLANATION OF PLATES

The photographs were all made by Mr. Joseph B. Haulenbeek.

## PLATE 21

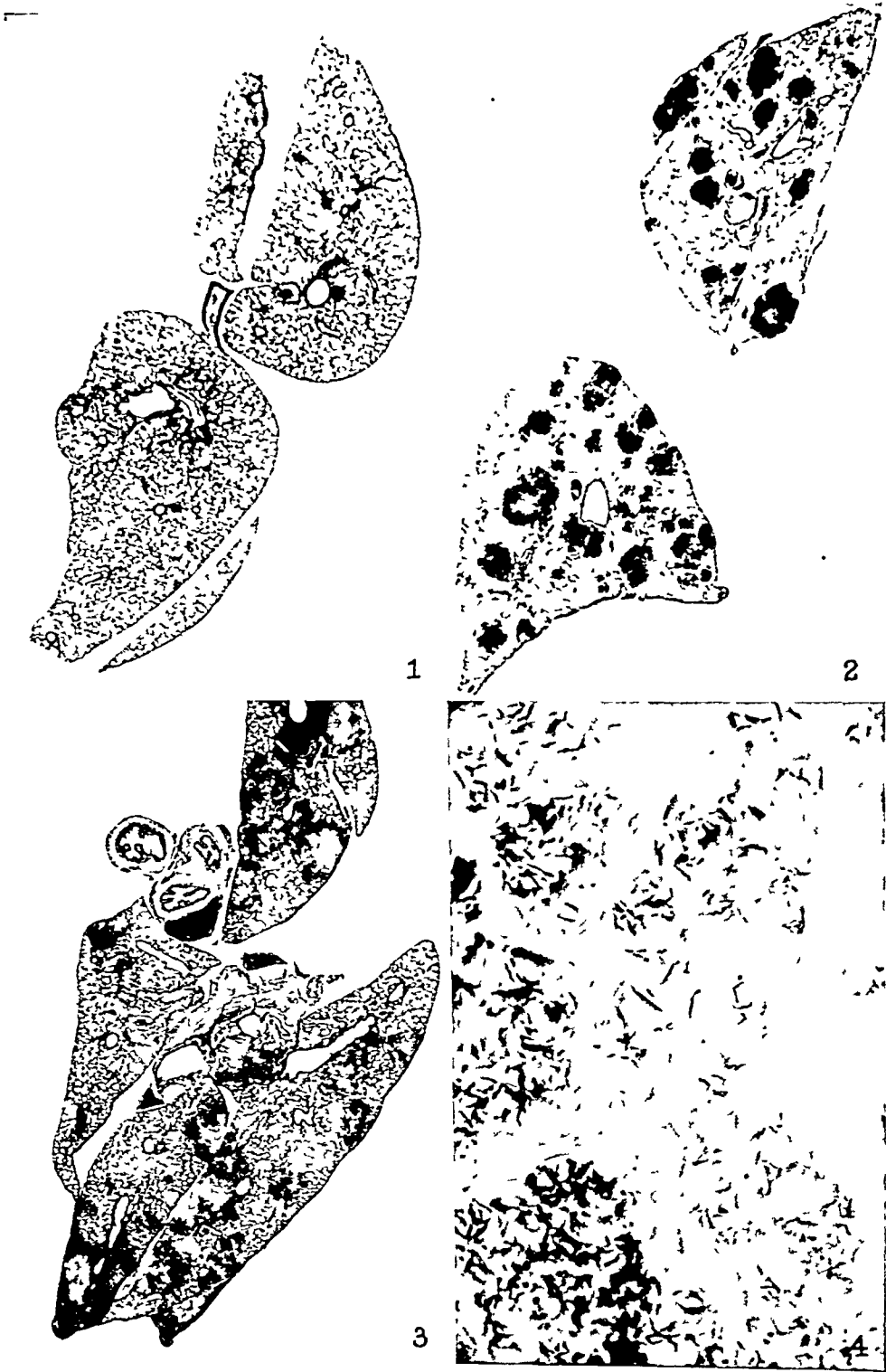
FIG. 1. Section of the lung of a mouse which was given tubercle bacilli intraperitoneally 6 weeks previously. Hematoxylin and eosin stain. Note absence of gross tuberculous lesions (score = 0).  $\times 9$ .

FIG. 2. Section of the lung of a mouse of the same strain which was given tubercle bacilli intraperitoneally and PVM intranasally 3 weeks previously. Hematoxylin and eosin stain. Note numerous large tuberculous lesions (score = +++).  $\times 9$ .

FIG. 3. Section of the lung of a mouse which was given tubercle bacilli intraperitoneally 6 weeks previously and PVM intranasally 3 weeks later. Hematoxylin and eosin stain. Note large tuberculous lesions (score = +++).  $\times 9$ .

FIG. 4. Section of a tubercle in a mouse similar to that shown in Fig. 2. Ziehl-Neelsen stain. Note large number of acid-fast bacilli.  $\times 1000$ .



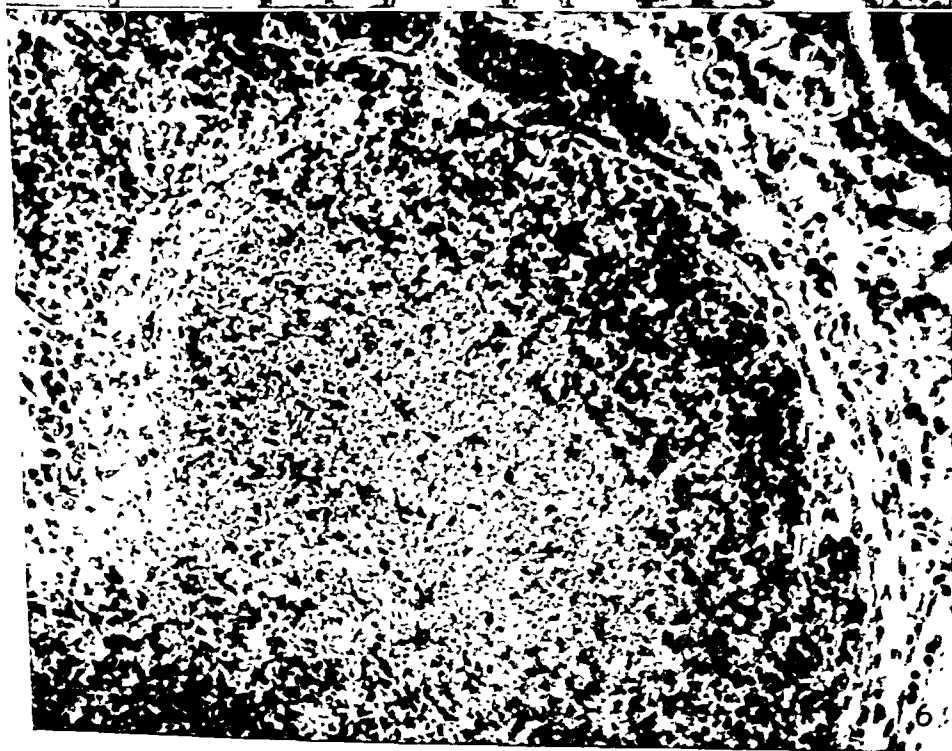
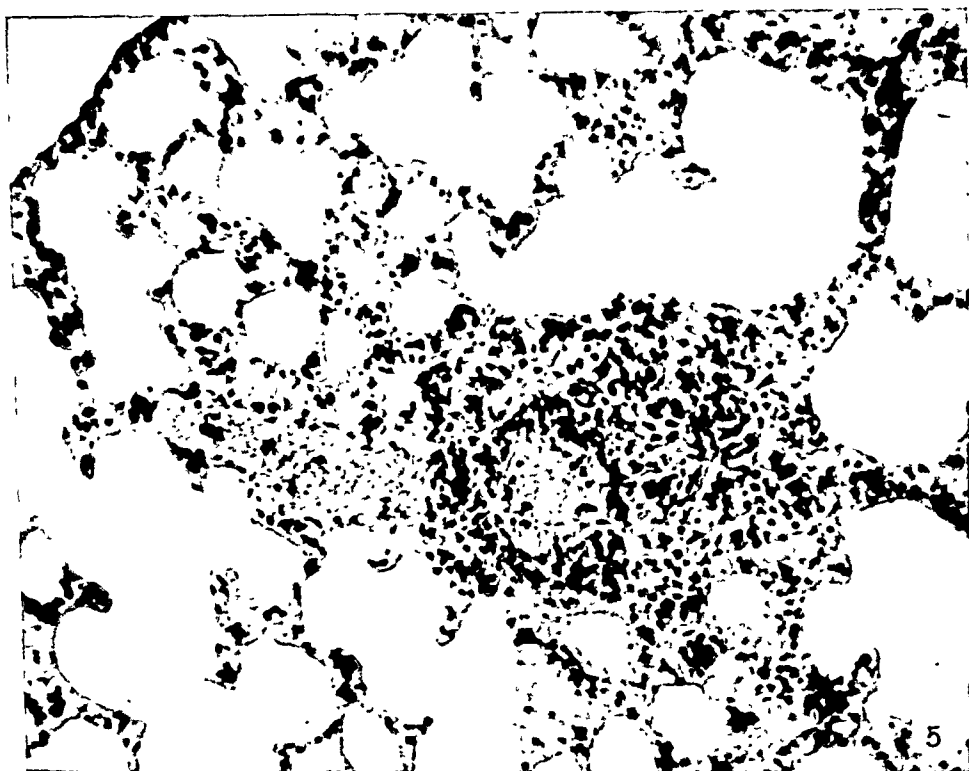


(Volkert *et al.* Concurrent infection in mice)

PLATE 22

FIG. 5. Section of a submiliary tubercle in the lung of a mouse which was given tubercle bacilli intraperitoneally 6 weeks previously. Hematoxylin and eosin stain.  $\times 275$ .

FIG. 6. Section of a tubercle in the lung of a mouse which was given tubercle bacilli intraperitoneally and PVM intranasally 3 weeks previously. Hematoxylin and eosin stain. Note central necrosis.  $\times 275$ .





# THE BINDING OF FATTY ACIDS BY SERUM ALBUMIN, A PROTECTIVE GROWTH FACTOR IN BACTERIOLOGICAL MEDIA

By BERNARD D. DAVIS,\* M.D., AND RENÉ J. DUBOS, PH.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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It has been reported from this laboratory that while polyoxyethylene sorbitan monooleate (marketed under the trade mark Tween 80<sup>1</sup>) facilitates submerged growth of tubercle bacilli in liquid media, initiation of growth by small inocula requires addition of serum albumin (1, 2). Other workers, using media without Tween, had previously noted that this protein promotes growth of tubercle bacilli (3, 4); in addition, whole serum has been employed for many years in a variety of media designed for the cultivation of tubercle bacilli and other bacteria.

At first we assumed that albumin, like most known growth factors, contributed a nutritive which was absorbed by the tubercle bacilli. After studying the mechanism of action of albumin, however, it was reported briefly (5) that some improvement in growth of small inocula was provided by the addition to the medium of albumin within a cellophane bag, even though this prevented contact between the bacteria and the protein; addition to the medium of the dialysate of albumin, however, was without effect. These observations were taken to indicate the presence in the medium of a dialyzable inhibitor which was bound to albumin. Subsequent work, published in a preliminary note (6), showed that the chief inhibitor against which albumin protects the tubercle bacilli is unesterified oleic acid present in the commercial product Tween 80, and further released from it during incubation.

Since oleic acid was not found to dialyze freely through cellophane, the interpretation of the earlier dialysis experiments is open to some doubt. Nevertheless, the conclusion, drawn from them, that albumin acts protectively by binding, not only is confirmed but is much more strikingly demonstrated by experiments involving albumin and oleic acid. These latter experiments will be described in the present paper, along with evidence that Tween 80, when freed of unesterified oleic acid, is not only non-toxic but even protective.

## EXPERIMENTAL

### *Methods*

Bacteriological experiments were performed as previously described (2), using 5 ml. of medium in a metal-capped wide test tube (25 × 250 mm.). The medium consisted of a

\* Tuberculosis Control Division, United States Public Health Service.

<sup>1</sup> Furnished through the courtesy of the Atlas Powder Company, Wilmington.

mineral mixture buffered by phosphate at pH 7.0, enzymatic casein hydrolysate, Vegex (yeast autolysate), and Tween 80 (0.05 per cent unless otherwise specified). After autoclaving at 120° for 10 minutes and addition of sterile glucose, 5 per cent neutralized bovine serum albumin (fraction V) (7), sterilized by filtration, was added aseptically to yield a final concentration of 0.1 per cent (unless otherwise specified). The other protein solutions tested were also sterilized by passage through sintered glass filters (Corning UF) and added aseptically. After inoculation the tubes were incubated at 37°C. The inoculum was a standard laboratory strain of a human tubercle bacillus, H37Rv, which had been grown for many passages in this Tween-albumin medium. The size of the inocula designated in the tables represents volumes of a 7 to 10 day old culture containing approximately 1 mg. of moist organisms (0.2 mg. dry weight) per ml. Growth is recorded in the tables in terms of a visual estimate ranging from 0 (no visible growth) to ++++ (full growth, approximately 2 mg. moist weight per ml.).

The oleic acid used was a commercial preparation (Eimer and Amend). The bovine serum albumins (fraction V and crystalline albumin) were obtained from the Armour Laboratories.<sup>2</sup>

### *Promotion of Growth by Undenatured Albumin*

The favorable effect of serum albumin on the growth of small inocula of the tubercle bacillus was not produced by comparable concentrations of a variety of other proteins: serum globulins (fractions II, III-1, and IV-1), gelatin, protamine, casein, ovalbumin, gliadin, or edestin. A very slight effect was exerted by  $\beta$ -lactoglobulin. Growth was promoted by serum albumin following dialysis, but not by its dialysate or by serum albumin following trypsin hydrolysis (sufficient to remove approximately 90 per cent of its precipitability by trichloroacetic acid); in this connection appropriate control experiments were carried out to determine that the loss of growth promotion following trypsin hydrolysis was not caused by any bacteriostatic effect of the trypsin or the hydrolysate. Finally, the property of facilitating growth was shown to be destroyed following heating of 5 per cent albumin to 100°C., whether the albumin was coagulated or was heated under conditions which avoided coagulation (neutralized, no salt added). Heating to 56°C. for 30 minutes, however, did not destroy the property. Some of these experiments are presented in Table I.

It was concluded that the binding property depends upon the native, undenatured configuration of the albumin molecule. Since it seemed unlikely that a bacterium could derive specific nutritive benefit from a whole protein molecule, the thought arose that the albumin, which is known to bind a variety

<sup>2</sup> Crystalline hen ovalbumin was furnished through the kindness of Dr. Gertrude Perlmann, and crystalline  $\beta$ -lactoglobulin (prepared by Dr. G. Haugaard) through the kindness of Dr. William Stein, of The Rockefeller Institute for Medical Research. The products of human plasma fractionation employed in this work were furnished through the kindness of the Department of Physical Chemistry, Harvard Medical School, and were developed under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

of small molecules (8-10), might be functioning as a protective rather than a nutritive growth factor.

*Protection by Albumin against Oleic Acid*

When it was found that 3 per cent of the oleic acid in commercial Tween 80 (0.6 per cent of the Tween by weight) is unesterified (6, 11) and that the tubercle bacillus is sensitive to extremely low concentrations of free oleic acid (12)

TABLE I

*Effect of Various Proteins on Growth of Tubercle Bacilli in a Medium Containing 0.05 Per Cent Unpurified Tween 80*

Substance added	Concentration	Inoculum (moist weight)	
		10 <sup>-3</sup> mg.	10 <sup>-4</sup> mg.
		Growth at 15 days	
	<i>per cent</i>		
Control.....		0	0
Bovine crystalline albumin.....	0.2	++++	++
Bovine albumin (fraction V).....	0.2	++++	++
Trypsin-digested bovine albumin.....	0.2	0	0
Trypsin-digested bovine albumin.....	0.5	0	0
Trypsin-digested bovine albumin + undigested bovine albumin.....	0.2		
	+0.2	++++	++
Dialyzed bovine albumin.....	0.2	++++	++
Human albumin (fraction V).....	0.2	++++±	±±
Human globulin (fraction II).....	0.2	0	0
Human globulin (fraction II).....	0.5	0	0
Ovalbumin.....	0.2	0	0
Ovalbumin.....	0.5	0	0
Bovine albumin heated to 56°.....	0.2	++++	++
Bovine albumin heated to 100°.....	0.2	0	0

(small inocula being sensitive to less than 1  $\mu$ g. per ml.—cf. Table IV), experiments were undertaken to test whether oleic acid might be the substance against which albumin protects the tubercle bacillus. Table II shows that albumin in a wide range of concentration will protect very small inocula of tubercle bacilli against oleic acid up to 1 per cent of the weight of albumin, and moderate inocula against 2 per cent. From these results it can be calculated that the concentration of albumin present in the usual medium (0.1 per cent) is easily capable of protecting the organism against the concentration of oleic acid (3  $\mu$ g. per ml.) which is introduced by the addition to the medium of 0.05 per cent Tween 80.

Albumin similarly protected tubercle bacilli against the toxic effect of added stearic acid.

TABLE II  
*Protection by Serum Albumin against the Bacteriostatic Effect of Oleic Acid on Tubercle Bacilli*

Oleic acid	Albumin	Inoculum (moist weight)		
		10 <sup>-2</sup> mg.	10 <sup>-4</sup> mg.	10 <sup>-6</sup> mg.
		Growth at 1½ days		
per cent	per cent			
Medium containing 0.05 per cent Tween 80				
0	0.1	+++++	+++++	++
0.001	0	+++++	0	0
	0.05	+++++	+++±	0
	0.1	+++++	+++++	0
0.002	0.05	+++++	0	0
	0.1	+++++	++	0
	0.2	+++++	+++++	+
0.004	0.1	+++++	0	0
	0.2	+++++	+++	0
	0.4	+++++	+++++	++
0.008	0.2	0	0	0
	0.4	+++++	+++	0
	0.8	+++++	+++++	+±
	1.6	+++++	+++++	+±
0.016	0.4	—	0	0
	0.8	+++++	+++	—
	1.6	+++++	+++++	0
Medium without Tween 80				
0.004	0.1	0	0	0
	0.2	+++++	+++	+
	0.4	+++++	+++	+

Growth in the medium without Tween was flocculent, so the figures are not comparable to those obtained in the medium with Tween.

#### *Elimination of Bacteriostatic Effect of Tween 80 by Purification*

Although the bacteriostatic property of the commercial product Tween 80 could thus be accounted for by its content of free oleic acid, direct proof that the Tween 80 molecule itself is not bacteriostatic required the preparation of material essentially free from fatty acid. The method of purification is de-



scribed in another paper (11); it yields a product containing less than 0.1 per cent unesterified oleic acid by weight. Table III shows that this purified Tween 80 is non-toxic for tubercle bacilli in high concentrations. From the data of Table IV, in the next section, it may be seen that inocula as small as  $10^{-7}$  mg. will grow in the presence of 0.05 per cent purified Tween 80 without addition of albumin.

The purification of Tween 80 has been indispensable for elucidating the mechanism of action of albumin, but it is not anticipated that the use of purified Tween 80 will replace the inclusion of albumin in the medium for most

TABLE III  
*Elimination of Bacteriostatic Effect of Tween 80 by Purification*

Tween 80  per cent	Inoculum (moist weight)		
	$10^{-3}$ mg.	$10^{-4}$ mg.	$10^{-5}$ mg.
	Growth at 9 days		
	Unpurified Tween, no albumin		
0.05	+++	++	±
0.1	+++	+	0
0.2	+++	0	0
0.4	+++	0	0
0.8	+++	0	0
	Fatty acid-free Tween, no albumin		
0.05	+++	++	—
0.1	+++	++	±
0.2	+++	++	±
0.4	+++	++	±
0.8	+++	++	±
	Unpurified Tween, 0.1 per cent albumin		
0.05	+++	++	±

purposes. Not only is it difficult to avoid traces of fatty acids, but the spontaneous hydrolysis of Tween 80 (11) is so fast that minimal inocula ( $10^{-8}$  mg. moist weight, 2 to 3 cells) cannot develop in the absence of albumin, even with freshly purified Tween 80.

While the bacteriostatic action of Tween 80 on the tubercle bacillus is entirely explained by its content of unesterified oleic acid, there are unknown bacteriostatic factors in some of the other Tweens. Several lots of Tween 60, for example, the analogous ester of stearic acid, yielded barely detectable amounts of free fatty acid (approximately  $\frac{1}{6}$  as much as Tween 80), yet these lots varied widely in their inhibitory effect on small inocula of the tubercle bacillus. It was determined that added stearic acid was quantitatively recovered from Tween 60 solution by the analytical method, and that stearic acid is less toxic

to the tubercle bacillus than oleic acid. The toxic effect of certain lots of Tween 60, then, is not due to their content of free fatty acid. It may be noted here that hydrogen peroxide is used in the manufacture of the Tweens.

TABLE IV

*Protection by Tween 80 against the Bacteriostatic Effect of Oleic Acid on Tubercle Bacilli*

Fatty acid-free Tween 80	Oleic acid	Inoculum (moist weight)					
		10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup> mg.
		Growth at 15 days					
per cent	μg./ml.						
No albumin							
0	0	++	+	+	± -	0	0
	1	0	0	0	0	0	0
	3	0	0	0	0	0	0
	10	0	0	0	0	0	0
0.01	0	—	+++	++	++	+±	+
	1	—	+++	++	+±	+	±
	3	+++±	±	0	0	0	0
	10	—	0	0	0	0	0
0.05	0	—	+++±	+++	++	++	++
	1	—	+++±	+++	++	+±	+
	3	+++±	+++±	+++	+±	+±	±
	10	0	0	0	0	0	0
0.2	0	++++	++++	+++±	—	++	++
	1	++++	++++	+++±	++±	++	+
	3	—	++++	+++±	++	+±	+
	10	++++	±	0	0	0	0
0.1 per cent albumin							
0.05	0	++++	++++	++++	+++	++±	++

*Protection by Tween 80 against Oleic Acid*

The "fatty acid-free" Tween 80 actually retains approximately  $\frac{1}{10}$  the free fatty acid originally present; i.e., 0.06 per cent by weight of oleic acid (6, 11). Nevertheless, when the toxic concentration of this purified material was sought, it was found that even 0.8 per cent purified Tween 80 showed no inhibitory effect on growth (Table III), in contrast to the marked effect of the smaller amount of free fatty acid in 0.05 per cent unpurified Tween 80. This phenomenon was explained by the observation that Tween 80 itself offers a certain amount of protection against the inhibitory effect of free oleic acid (Table IV). The Tween 80 presumably combines reversibly with free fatty acid, as does serum albumin, but is less effective per unit concentration than the albumin.

Because of this protective effect, Tween 80 need not be absolutely free of unesterified acid in order to be effectively fatty acid-free for bacteriological work; it need only have a proportion of fatty acid to ester which is below a certain critical level. Baker, Harrison, and Miller (13) have likewise shown that an unionized surface active agent rather similar to the Tweens (Demal, a mixture of polyglycerol esters), and natural phospholipids as well, prevent inhibition of bacterial metabolism and sterilization by either cationic or anionic synthetic detergents.

That there is some sort of interaction between Tween and fatty acids could be predicted from the capacity of Tween to disperse large amounts of fatty acids and various other lipids in clear solution in water.

### *Effect of Albumin on Surface Growth*

When a tube of Long's synthetic medium (which contains no Tween) was inoculated for surface growth by transfer of a small piece of pellicle of the tubercle bacillus H37Rv grown for 4 weeks on the same medium, growth at the edge of the inoculum was first visible in 4 to 6 days. When 0.1 per cent serum albumin was added to this medium, pellicle growth was initiated much more rapidly, being visible within 2 to 3 days of inoculation. It appears likely that the delay and occasional failure in the initiation of surface growth on simply synthetic media may be due to contamination by traces of fatty acid. The delay may be accounted for by the observation that tubercle bacilli can absorb and presumably metabolize fatty acids in bacteriostatic concentrations, and then multiply after elimination of this excess of fatty acid (13 a).

The addition of oleic acid as well as albumin further slightly stimulated surface growth. This observation parallels the stimulating effect of long chain fatty acids, in the presence of albumin, on other modes of growth of tubercle bacilli, submerged in a liquid medium or on the surface of a solid medium (12).

It appears unlikely that the addition of albumin would be of practical value in the preparation of tuberculin, for which surface cultures of the tubercle bacillus are largely used, since the inclusion of a foreign protein would be undesirable and the use of very large inocula permits quite regular initiation of growth.

### *Binding Capacity of Serum Albumin for Oleic Acid*

(a) *Bacteriological*.—With fatty acid-free Tween 80 it was possible to study quantitatively the binding capacity of albumin for oleic acid in the medium. Table II showed that serum albumin is able, over a wide concentration range (0.1 to 1.6 per cent), to bind 1 to 2 per cent of its weight of oleic acid firmly enough to neutralize the bacteriostatic effect of the fatty acid. This corresponds to 3 to 6 molecules of oleic acid (molecular weight 282) per molecule of albumin (molecular weight 70,000).

While these bacteriological studies clearly suggest that serum albumin can bind oleic acid in the proportion indicated, an element of uncertainty arises from the possibility that (a) the ratio obtained may represent a competition for the surface of the bacteria, rather than a physicochemical equilibrium in homogeneous solution; and (b) albumin, oleic acid, or impurities in either may have unknown effects on the metabolism of the tubercle bacillus which influence the result. The interaction between albumin and oleic acid was therefore studied by two further methods: inhibition of the hemolytic effect of oleic acid on red blood cells, and inhibition of the opalescence of emulsions of oleic acid.

(b) *Hemolytic*.—Table V shows that serum albumin protects red cells from hemolysis by oleic acid, and that the amount of fatty acid bound by the albumin is between 2 and 4 per cent, somewhat larger than that observed in the bacteriological experiments described above. Crystalline  $\beta$ -lactoglobulin protected the red cells from  $\frac{1}{2}$  to  $\frac{1}{4}$  as much oleic acid as did serum albumin; ovalbumin, protamine, and gelatin offered no protection.

Tween 80 proved to be approximately as effective as albumin in protecting red cells from hemolysis during incubation with added oleic acid for 30 minutes at 37°C.; however, Tween 80 itself was very hemolytic when the incubation was continued overnight in the refrigerator.

No further effort was made to refine the hemolytic method, which may be capable of much greater precision than is indicated by Table V. It was observed that sheep red cells demand a rather large amount of oleic acid (approximately 1 per cent of the weight of cells) to produce hemolysis. The sensitivity of the method as a test for unbound oleic acid is consequently greater the lower the concentration of red cells.

(c) *Chemical*.—It was not found possible to attain dialysis equilibrium with oleic acid across a cellophane membrane; the binding of oleic acid by albumin could therefore not be measured by this method, as had been done with various other compounds bound by albumin (8, 14, 15). Another thermodynamically sound method of measuring the interaction, however, is the increase in solubility of oleic acid in the presence of albumin. Since exceedingly small amounts of sodium oleate become opalescent when added to a neutral phosphate buffer, the binding capacity of serum albumin may be simply estimated by measuring the ratio of oleic acid to albumin which produces opalescence at various albumin concentrations.

This principle is illustrated in Table V, which indicates not only the effect of albumin on hemolysis, but the clarity or opalescence of the mixtures of oleate and albumin before addition of red cells. It is seen that the binding capacity of albumin is between 2 and 4 per cent, the variation depending on the roughness of serological dilutions. Further experiments indicated a binding capacity of approximately 3 per cent, as judged by the extinction of opalescence. This value corresponds to a ratio of approximately 9 molecules of oleic acid (molecular

weight 282) per molecule of serum albumin (molecular weight 70,000). It may be noted that the binding capacity of albumin observed in the bacteriological tests was only 3 to 6 molecules of oleic acid. The discrepancy is not surprising, since (1) the bound oleic acid is undoubtedly in equilibrium with free acid, the concentration of free acid increasing with increasing saturation of the protein, and (2) the bacteria are apparently sensitive to a lower concentration of free acid than that found in an aqueous solution in equilibrium with an excess of solute (as indicated by opalescence).

TABLE V

*Protection by Serum Albumin against Hemolysis by Oleic Acid*

Fresh sheep red blood cells were washed 4 times in neutral phosphate-saline buffer (0.14 M NaCl, 0.02 M phosphate, pH 7.0), and suspended in this buffer in a concentration of 0.4 per cent by volume. A 1 per cent solution of oleic acid in dilute NaOH was neutralized with concentrated HCl to incipient turbidity. Bovine serum albumin (fraction V) was neutralized in 5 per cent solution. Dilutions of the albumin and the oleate solutions were prepared in the phosphate-saline buffer. Volumes of 0.5 ml. of the albumin dilutions were added to 1.0 ml. of the oleate dilutions in small test tubes; 0.5 ml. of the red cell suspension was added, and the tubes were incubated in a 37°C. water bath for 30 minutes. In this experiment the results indicated were unchanged after further incubation overnight in the refrigerator, although in some other experiments certain borderline tubes developed hemolysis overnight. The concentrations noted are the final concentrations in the mixtures. Also indicated in the table is the clarity or opalescence of each mixture of albumin and oleic acid before addition of red cells.

Concentration of albumin per cent	Concentration of oleate, per cent				
	0.05	0.02	0.01	0.005	0.002
1.0	0(cl)	0(cl)	0(cl)	0(cl)	0(cl)
0.5	H(op)	H(op)	0(cl)	0(cl)	0(cl)
0.25	—	—	0(cl)	0(cl)	0(cl)
0.1	—	—	H(op)	H(op)	0(cl)
0.05	—	—	H(op)	H(op)	0(cl)

H = hemolysis.

0 = no hemolysis.

op = opalescent mixture of oleate and albumin.

cl = clear mixture of oleate and albumin.

Serum albumin has a greater capacity for tightly binding long chain fatty acids than any other substances tested. Human serum globulin (fraction II), protamine, gelatin, and crystalline ovalbumin, tested in 3 per cent solution, had no effect on the opalescence of oleic acid. This absence of binding explains the earlier observation that these proteins exerted no beneficial effect on the cultivation of tubercle bacilli. Crystalline  $\beta$ -lactoglobulin quenched the opalescence of approximately half as much oleic acid per unit weight of protein as did serum albumin.

## DISCUSSION

It has been established that the predominant if not the only rôle of serum albumin in permitting initiation of growth of tubercle bacilli by small inocula is to function as a protective rather than a nutritive growth factor.<sup>3</sup> In the media under present consideration the growth inhibitor bound by the albumin is free oleic acid, largely contributed by the Tween 80. Similarly, Gould, Kane, and Mueller (17) have recently reported that starch promotes growth of gonococci on solid media by binding traces of an inhibitor present in the agar; this inhibitor was found by Ley and Mueller (18) to be oleic acid. It is now possible to interpret in terms of a protective growth factor the older observation of Uyei (19) that the value of potato extract in media for the diagnostic cultivation of tubercle bacilli is due to the soluble starch present in this extract. The affinity of starch for fatty acids (20) is probably less than that of albumin, for we have not found starch nearly as effective as albumin in promoting growth of tubercle bacilli in solid media containing Tween 80.

The binding property of serum albumin undoubtedly contributes to the value of blood, serum, or ascitic fluid in various "enriched" media. The commercial availability of pure serum albumin now permits better controlled utilization of this property. Since fatty acids are ubiquitous contaminants of glassware, cotton plugs, and reagents, and since many bacteria (particularly Gram-positive and acid-fast species) are very sensitive to fatty acids, it appears probable that the property of albumin here described will be useful in obtaining reproducible growth or growth from minimal inocula of organisms other than the tubercle bacillus. In the field of microbiological assay of vitamins and amino acids, for instance, it has been pointed out that irregularities in the growth response of *Lactobacillus casei* are frequently caused by contamination by fatty acids (21, p. 85). Indeed, even outside the domain of bacteriology it has been observed by Clarke (22) that commercial Tween 80 depresses the respiration of red blood cells parasitized by *Plasmodia*; the toxic effect may either be corrected by the addition of serum albumin or avoided by the use of fatty acid-free Tween 80.

The reversible binding of fatty acids by albumin permits the fatty acids to serve as nutrient growth factors which enhance the growth of tubercle bacilli (especially of the avian type) and other bacteria (12), whereas in the absence

<sup>3</sup> The initiation of growth is facilitated equally well by crystalline bovine serum albumin, but the less pure amorphous fraction V also has a further slight enhancing effect on the richness of growth which has been traced to a heat-stable, dialyzable factor (12). In addition to its desirable effects, fraction V has the undesirable property of rendering the medium unstable through the contamination of the albumin by a trace of lipase, which slowly hydrolyzes the Tween and releases free oleic acid. The lipase may be inactivated by heating the albumin at 56°C. for 30 minutes, which does not destroy the desirable binding property of the albumin, or by adding NaF. The albumin then permits regular initiation of growth by smaller inocula (two bacterial cells) than have otherwise been effective (16).

of albumin the fatty acid is bacteriostatic. The albumin apparently "buffers" the fatty acid so that the concentration of free fatty acid is very low, below the bacteriostatic level, while the bound acid constitutes a reserve which replaces the fatty acid withdrawn by the bacteria. Esterification of the fatty acid plays a similar rôle, except that it appears possible that the bacterium absorbs the free fatty acid in the one case, and the ester in the other (16).

In this study serum albumin has served as a useful chemical reagent, now conveniently available as a result of the wartime plasma fractionation program. But the implications of the results presented here are not limited to the field of microbial nutrition, for albumin is a substance of biological origin. The relative uniqueness of this property of albumin must be stressed,  $\beta$ -lactoglobulin being the only other protein found so far to show even a fraction of the same affinity. This uniqueness has also been reported for the weaker interaction of albumin with short chain fatty acids (23), and is probably true of its interactions with many other organic anions, including sulfonamides (14), anionic dyes (15), and a variety of drugs and other compounds (reviewed in 8-10). Since fatty acids are physiologically the most important members of the group of bindable substances studied thus far, considerations of teleology (or, more precisely, of evolutionary survival value) suggest that this property of albumin probably serves a useful physiological function, such as transport of materials in the blood and protection of animal cells against toxic effects of various substances (*e.g.*, hemolysis by free fatty acids). The combination of cytotoxic and nutritive properties of fatty acids, and the modification of the balance of these properties by serum albumin, may find quite a close analogy in the animal organism to the effects observed in bacteriological culture media.

The physiological significance of the binding capacity of serum albumin has been discussed in greater detail elsewhere (10). The affinity of serum albumin for chemotherapeutic agents with anionic groups is of special practical importance. The failure of a large number of bacteriostatic substances (including fatty acids) to be effective chemotherapeutic agents *in vivo* can be attributed partly to their affinity for serum albumin. One cannot overemphasize the importance of including serum or albumin in the medium when testing potential chemotherapeutic agents *in vitro*. The inefficacy of penicillin K *in vivo*, for instance, as compared with penicillins G, F, and X, (all having been standardized *in vitro* in the absence of albumin), has recently been accounted for by the much greater extent to which serum albumin binds penicillin K (24, 24 a). In connection with the present work it is of interest to point out that the penicillins consist essentially of a hydrocarbon chain or ring separated by a pair of unusual heterocyclic rings from a carboxyl group. Penicillin K, which is bound approximately 8 times as extensively as penicillin dihydro F, differs from the latter simply by having two more carbon atoms on its hydrocarbon chain; *i.e.*, resembling a longer chain fatty acid.

Although the inhibitory effect of serum on the antibacterial and hemolytic action of soaps was observed at least as early as 1907 (25-27), and the interaction of proteins with drugs, indicator dyes, etc., has been known to biologists for decades, it is only in very recent years that these reversible reactions have begun to be seriously used as physicochemical tools for studying protein structure (15, 28). Our knowledge of the structures responsible for these reactions is as yet exceedingly scanty. The failure of certain proteins to bind oleic acid cannot be ascribed to the lack of any known components of serum albumin. The special capacity of albumin therefore implies the presence on the surface of the molecule of regions where the specific configuration of the amino acid residues leads to interaction with the fatty acid. This view is supported by the fact that binding demands the native, undenatured configuration of the albumin.

The type of configuration which might be predicted to be suitable for binding a long chain fatty acid would be a quaternary nitrogen atom, binding the oppositely charged carboxyl ion, adjacent to several non-polar residues which would attract the non-polar chain of the fatty acid by van der Waals forces. Since the most distinctive feature of the composition of serum albumin is its unusually high content of lysine and leucine, it is tentatively suggested that albumin may have a number of lysine residues each of which is adjacent to several leucine or other non-polar residues. (For a similar suggestion, *cf.* 29.) The importance of the regions adjacent to the quaternary nitrogen is emphasized by the absence of binding by the basic protein protamine, which contains approximately 90 per cent arginine and hence an abundance of quaternary nitrogens.

The assumption that the quaternary nitrogen of lysine is involved in the binding is strengthened by the observation of Klotz (28) that pH affects the binding of anionic dyes by albumin only in the region (above pH 9) where the  $\epsilon$ -amino nitrogen of lysine loses its charge. Only a fraction of the approximately 60 lysine groups of albumin would be needed to account for the binding of 9 oleic acid molecules. Indeed, until further information is obtained, we cannot be certain that as many as 9 binding sites are present on each albumin molecule, for the tendency of fatty acids to form micelles in aqueous solution may also apply to their interaction with albumin. This seems unlikely, however, in the very dilute solutions under consideration. The binding of shorter chain fatty acids by albumin, although less tight, is also reported to involve at least 9 molecules, in ultrafiltrates from 0.005 M caprylate (30).

It must be emphasized that the data reported here are crude, judged by the standards of physical chemistry, and serve to measure only the order of magnitude of the interaction. Precise chemical experiments did not seem warranted at this stage, since our purpose was to compare the chemical results with the bacteriological data, which had already been obtained with commercial oleic



acid, a notoriously impure product, and with Tween 80 manufactured from such material. More precise experiments on the binding of long chain fatty acids by serum albumin are planned.

#### SUMMARY

Serum albumin is a protective bacterial growth factor; by binding traces of fatty acid in the media it permits initiation of growth by the smallest possible inocula of tubercle bacilli. Each molecule of albumin binds 3 to 6 molecules of oleic acid (1 to 2 per cent of the weight of the albumin) tightly enough to prevent bacteriostasis, and 9 molecules of oleic acid in equilibrium with a saturated neutral solution. The property requires undenatured albumin. Crystalline  $\beta$ -lactoglobulin has a smaller capacity, and a number of other proteins no perceptible capacity to bind oleic acid.

The inhibitory effect of the commercial product Tween 80 (polyoxyethylene sorbitan monooleate) on the growth of small inocula of tubercle bacilli in liquid media is caused by its content of unesterified oleic acid (0.6 per cent by weight). Purified Tween 80, freed of this contaminating fatty acid, not only permits growth of small inocula, but protects against small amounts of added oleic acid.

The implications of the binding capacity of albumin for its possible physiological significance in the animal body (transport; protection against cytotoxins), and for the structure of the protein, are briefly discussed.

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## ST. LOUIS ENCEPHALITIS

### TRANSMISSION OF VIRUS TO CHICKENS BY INFECTED MITES *DERMANYSSUS GALLINAE* AND RESULTING VIREMIA AS SOURCE OF VIRUS FOR INFECTION OF MITES\*

BY MARGARET G. SMITH, M.D., RUSSELL J. BLATTNER, M.D.,  
AND FLORENCE M. HEYS, Ph.D.

(From the Department of Pathology and the Department of Pediatrics, Washington University School of Medicine, St. Louis)

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Chicken mites (*Dermanyssus gallinae*) collected in several localities in St. Louis County have been found to be infected with the virus of St. Louis encephalitis (1, 2). The infection is probably permanent since congenital transfer of the virus in these mites has been demonstrated. Persistence of the virus in nature is indicated likewise by isolation of the virus from mites collected at a single site at intervals of 6, 8, and 16 weeks and by persistence of the virus for many months in laboratory colonies, one established with mites found infected in nature (31 months) and one established with experimentally infected mites (21 months). Experimental infection of mites from a homogeneous colony of uninfected mites derived from a single female, was accomplished by allowing them to feed on chickens having viremia following subcutaneous inoculation of the virus. Transovarian passage in these experimentally infected mites has been demonstrated (3).

However, before these observations can be considered significant in the epidemiology of St. Louis encephalitis, it is necessary to show that infected mites are capable of transferring the virus of St. Louis encephalitis to chickens and that such chickens can serve as the source of virus for a blood-sucking vector. The present paper reports findings which show that infected mites, both those found infected in nature and those infected experimentally in the laboratory, are capable of producing viremia in chickens by bite and that these chickens in turn can serve as a source of the St. Louis virus for the infection of mites.

### Materials

*Mite Colonies Free of the St. Louis Encephalitis Virus.*—The colonies of uninfected mites (*Dermanyssus gallinae*) used in this investigation were subcolonies from a homogeneous parent colony, shown by repeated tests to be free from the virus of St. Louis encephalitis. The establishment of this original homogeneous colony from a single adult female and her nymph offspring has been described in a previous communication (3).

*Mite Colonies Infected with the St. Louis Encephalitis Virus.*—Four colonies of chicken

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mites have been used: one colony derived from mites found infected in nature with the St. Louis virus (Rippy strain), and three colonies of mites infected experimentally in the laboratory with three strains of St. Louis encephalitis virus. The colony derived from mites found infected in nature has been maintained in the laboratory since October 5, 1944, by feeding on normal chickens. The colonies of experimentally infected mites were established with mites from the homogeneous uninfected colonies after they had been allowed to feed upon chickens inoculated subcutaneously with strains of the St. Louis virus. The method of infecting mites and the testing of these mites for the presence of virus have been described previously (3). Mites were infected experimentally with three different strains of the St. Louis virus: (a) the Rippy strain (RN<sub>6</sub>), a mouse brain virus isolated from mites found infected in nature; (b) the Hubbard egg membrane strain, an egg membrane strain of St. Louis virus (Hubbard) isolated in mice from human brain tissue in 1937 and maintained since 1938 on the chorioallantoic membrane of the developing hen's egg (4); (c) the Mullen strain, isolated in mice in 1945 from the blood of a patient who recovered (5).

*Chickens.*—The chickens used in these experiments were New Hampshire Reds, 8 to 20 days of age. All chickens were hatched in the laboratory and carefully protected at all times from possible exposure to arthropods.

### *Transmission of the Virus of St. Louis Encephalitis to Chickens by Infected Mites*

Many preliminary attempts to demonstrate viremia in chickens following the bite of infected mites were made by direct inoculation (0.03 to 0.04 ml.) of serum or whole blood intracerebrally into young Swiss mice. In no instance did either serum or whole blood produce any signs suggestive of encephalitis. Previous experience (6) had shown, however, that small amounts of the St. Louis encephalitis virus, insufficient in quantity to produce signs of encephalitis in mice even by the intracerebral route, could increase sufficiently by chorioallantoic passage in the developing hen's egg to produce signs of encephalitis when the egg membrane material was transferred intracerebrally to mice. In the light of this experience it seemed possible that virus might be present in the blood of chickens on which infected mites had fed but not in sufficient quantities to be detected by the direct inoculation of mice.

Accordingly, in a further series of experiments heparinized blood drawn from the heart of chickens at intervals after the beginning of the period during which the infected mites had fed, was injected intracerebrally into Swiss mice (0.03 ml.), and simultaneously 0.03 to 0.1 ml. of the same blood sample was inoculated on the chorioallantoic membrane of the developing hen's egg. Four days after inoculation these membranes were harvested and ground with a small amount of tryptose phosphate broth. The resulting suspension was centrifuged at low speed for 2 minutes, and the supernatant fluid was passed to a second series of embryonated eggs and at the same time was inoculated intracerebrally in 0.03 ml. amounts into white Swiss mice. As before, the passage membranes were harvested after a 4 day incubation period and were ground with broth. After centrifugation, the supernatant fluid was injected intracerebrally in 0.03 ml. amounts into white Swiss mice.

The colony of infected mites used in this series of experiments was derived from mites found infected in nature, the so called Rippy colony. The first three chickens tested for viremia by the above method were bled as follows: one at 18, one at 21, and one at 26 hours following

contact with the Rippy colony of infected mites. Virus was isolated from the blood of each of these three chickens by egg membrane passage and subsequent intracerebral inoculation of mice, but in no instance did any of the mice injected directly with fresh heparinized blood show signs of illness.

A fourth chicken was exposed to the Rippy colony of infected mites in the same way as before. This chicken was bled on three occasions—at 48, 72, and 93 hours counting from the time that the mites first had opportunity to feed. Virus was isolated from the blood obtained at each of these intervals by egg passage technique. Results obtained by direct inoculation of mice were negative.

In order to obtain some idea concerning the duration of viremia, a fifth chicken was bled at 20, 48, 67, 97, and 115 hours after the infected mites were given opportunity to feed. Virus was isolated from the blood of this chicken at 20, 48, 67, and at 97 hours, but not at 115 hours. Here again in no instance were signs of encephalitis observed in mice injected with the fresh heparinized blood. However, after two egg passages the presence of the virus was demonstrated readily by the intracerebral inoculation of mice with egg membrane material. Details of this experiment are given in Fig. 1. By means of the same method combining egg and mouse inoculation, the blood of this chicken (No. 5) was tested again for virus at 18 days and at 32 days following the first exposure to infected mites. Both tests gave negative results. After the second of these negative bleedings (at 32 days) this chicken was placed in contact with the infected mites for a second exposure in the same manner as that described for the first exposure. On this occasion the chicken was bled at 24, 48, 67, 100, and 123 hours after the beginning of the feeding period. By means of chorioallantoic passage, virus was detected in blood samples drawn at 48 and 67 hours, but not in those drawn at 24, 100, and 123 hours. Thus viremia occurred a second time in this chicken, resulting apparently from reexposure to infected mites 32 days after the first exposure.

The virus isolated from blood drawn at the 48 hour period in each of the above series of bleedings was identified by mouse protection tests, using known St. Louis immune rabbit sera, as the virus of St. Louis encephalitis.

Subsequently, virus was isolated by the egg passage technique from fifteen other chickens after exposure to the naturally infected mites (Rippy colony). Results obtained in these experiments are shown in Table I.

#### *Viremia in Chickens Owing to the Bite of Experimentally Infected Mites*

The first attempt to demonstrate virus in the blood of a chicken exposed to the experimentally infected mites (RN<sub>6</sub> strain) was not successful even by chorioallantoic passage. However, in later experiments virus was isolated with regularity from the blood of eighteen chickens fed upon by experimentally infected mites. Such viremia was produced by mites infected with the Rippy strain (RN<sub>6</sub>) of virus and also with two other strains of the St. Louis virus, the Hubbard egg membrane strain and the Mullen strain, one isolated from the brain of a fatal case of encephalitis and the other isolated from the blood of a patient (4, 5). Three control experiments, in which the same procedures were followed but in which the chickens were exposed to mites free of the St. Louis virus, gave negative results. A summary of these results is included in Table I. During this phase of the experimental work many of the chickens tested

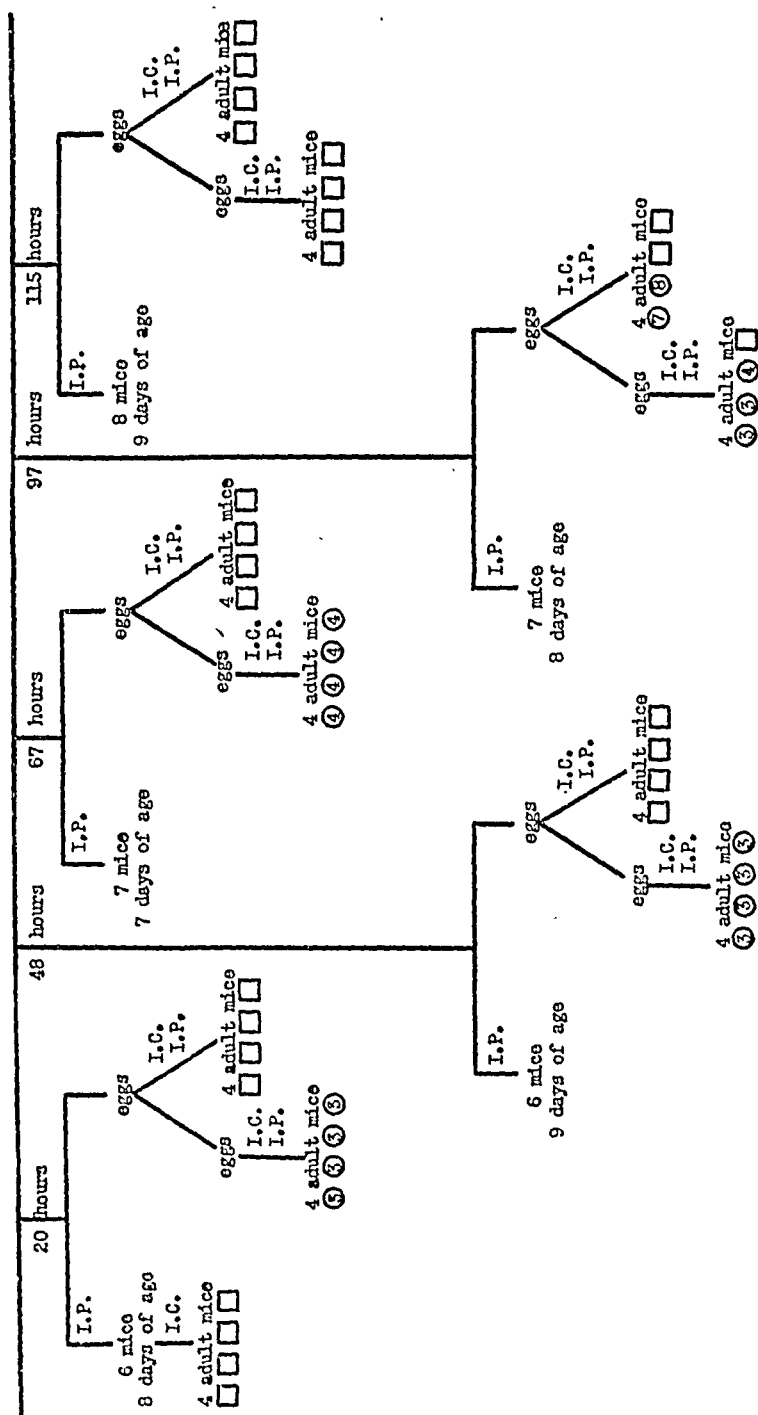


FIG. 1. Transmission of virus of St. Louis encephalitis to normal chickens by bite of infected chicken mites (*Dermanyssus gallinae*). Chicken, 20 days of age, fed upon by infected mites (Rippy colony) for period of 4 hours; heparinized blood drawn from heart at intervals following beginning of exposure period. Inoculations: 0.06 to 0.07 cc. heparinized blood on chorioallantoic membrane; 0.05 cc. heparinized blood injected intraperitoneally into young mice, 6 to 9 days of age.

for viremia were being used in connection with an investigation dealing with the mosquito transmission of the virus of St. Louis encephalitis.<sup>1</sup>

It is well to reemphasize that viremia in chickens bitten by infected mites is demonstrated with comparative ease by means of the chorioallantoic passage technique but that viremia has not been shown by the direct inoculation of serum or of fresh heparinized blood intracerebrally into mice. Multiplication

TABLE I

*Transmission of the Virus of St. Louis Encephalitis to Chickens by Bite of Infected Mites (Dermanyssus gallinae). Viremia Demonstrated by Chorioallantoic Passage*

Strain of virus infecting mites	No. of chickens tested	Viremia in chickens at intervals following the beginning of feeding by mites			
		24 to 30 hrs.	44 to 54 hrs.	72 to 77 hrs.	90 to 97 hrs.
Rippy strain (infected in nature)	3	0	+	+	+
	1	0	—	+	+
	6	0	+	0	0
	5	0	+	0	+
RN <sub>6</sub> strain	1	—	—	—	—
	1	+	+	+	0
	1	0	—	+	+
	2	0	+	0	+
	5	0	+	0	0
Hubbard egg membrane strain	1	0	+	+	+
	1	+	0	+	0
	2	0	+	+	0
Mullen strain	3	0	+	0	+
	1	0	+	0	0
	1	0	+	+	0
Control (normal mites)	3	0	—	0	—

+

 = virus present in blood.

—

 = virus not present in blood.

0

 = blood not tested.

of the virus by egg membrane passage seems to be required before its presence can be demonstrated by the intracerebral inoculation of mice.

#### *Chickens Bitten by Infected Mites, as a Source of Virus for the Infection of Other Mites*

Since it was evident that the amount of virus present in the blood of chickens following the bite of infected mites is small, it was of interest to ascertain

<sup>1</sup> Data to be published.

whether this small amount of virus circulating in the blood could serve as a source of virus for other (uninfected) mites.

When such a chicken infected by the bite of mites is used as a possible source of virus for uninfected mites, there is always the probability that mites from the infected colony remaining on the chicken will be carried over to the uninfected colony, defeating the purpose of the experiment at the outset. If, however, mites of the adult stage only are used to infect the chicken, and this chicken is exposed within the next 48 hours to mites of an uninfected colony, one can assume that all engorged first stage nymphs which are collected from the chicken must have come from the uninfected colony of mites constituting the second exposure. Nymph offspring of the adult infected mites which were used to infect the chicken cannot be present owing to the fact that insufficient time has elapsed for the development of nymphs from eggs laid by these infected adults, whereas nymphs found on the chicken are from the general uninfected colony composed of mites of all stages of development. Accordingly, the following experiments were carried out.

In order to obtain adult infected mites only, a normal chicken, 10 days of age, was placed in a container housing a colony of mites experimentally infected with the RN<sub>6</sub> strain of St. Louis virus. After 2 hours the chicken was removed and placed in a covered crock. During the next several hours, large engorged adult mites which dropped from the chicken were collected from the crock and placed in glass tubes. After egg laying the adults were separated from their eggs. Four or 5 days later the adult mites were placed on black paper, examined, and collected again in tubes. This procedure was followed in order to make sure that unfed nymphs which might have hatched from undetected eggs were eliminated. For each experiment 800 to 1000 adult infected mites were collected in the above manner, being placed each time in an autoclaved container.

The experimental procedure consisted in placing a normal chicken approximately 2 weeks of age in contact with these infected adult mites in the late afternoon and allowing exposure to continue during the night. Twenty-four hours after the beginning of this feeding period the chicken was used as a possible source of infection for uninfected mites. It was placed in a container housing a colony of uninfected mites, allowed to remain in contact with the mites for 2 hours in the late afternoon, and then removed to a covered crock. Mites remaining on the chicken continue to feed during the night. Approximately 16 hours later the chicken was removed, and the engorged mites of the smallest size, all believed to be first stage nymphs, were collected from the crock. These first stage nymphs were placed in glass tubes and allowed to moult to the second nymph stage. All of the engorged nymphs must have come from the uninfected colony since the mites used to infect the chicken were adults, and sufficient time had not elapsed for nymphs to have developed from eggs laid by these adults. Five to 7 days after these mites had been collected as engorged first stage nymphs, they were tested for virus.

In the course of four experiments of this type, details of which are given in the next paragraph, samples of approximately 150 to 920 mites were triturated in small amounts of tryptose phosphate broth and the resulting suspensions were inoculated intraperitoneally into young Swiss mice under 10 days of age.



These young mice were observed closely. While none developed definite convulsions, signs of illness such as roughening of the fur, unusual excitability or lethargy with failure to nurse appeared within 12 to 13 days after intraperitoneal inoculation. The brain of each mouse showing signs of illness was removed and passed by intracerebral inoculation to four or five adult mice. All the brains proved bacteriologically sterile on culture. In the experiments giving positive results the adult passage mice developed convulsions in 3 to 5 days following intracerebral inoculation. The infectious agent thus recovered from mites was identified in each instance as the virus of St. Louis encephalitis by means of the mouse protection test using the serum of a rabbit immunized with the virus of St. Louis encephalitis (Hubbard strain).

Results obtained in the four experiments are summarized briefly as follows:—

In the first experiment a suspension of approximately 850 nymphs was inoculated intraperitoneally into eight mice, 8 days of age. Two of the eight mice appeared ill on the 13th day following inoculation and virus was recovered from the brain of each. In a second experiment two samples of nymphs consisting of approximately 700 and 150 respectively were tested for virus. The suspension of 700 nymphs was inoculated intraperitoneally into six mice, 7 days of age. Two of the six began on the 12th day to show alternate periods of excitability and lethargy. Virus was recovered from the brain of each of these two by intracerebral passage to adult mice. The suspension of 150 nymphs was inoculated intraperitoneally into six mice, 12 days of age. One of the six showed definite signs of illness on the 12th day. It was killed on the 13th day and virus was recovered from the brain. A third experiment gave unsatisfactory results: Two of eight mice, 9 days of age, which had been inoculated intraperitoneally with a suspension of approximately 920 nymphs, were found dead and partially eaten on the 13th day. The brain of each of the other six young mice was tested for virus by intracerebral passage to adult mice with negative results. In a fourth experiment two samples of nymphs, 720 and 325, were tested for virus. The suspension of 720 nymphs was tested by intraperitoneal inoculation of seven mice, 8 and 9 days of age. Four of the seven mice appeared ill on the 12th and 13th days. Virus was recovered from the brain in the case of two of the four. The suspension of 325 nymphs in this experiment was tested by intraperitoneal inoculation of six mice, 8 to 9 days of age. Virus was recovered from the brain of each of two, which appeared ill on the 10th day.

Thus in three of four experiments the virus of St. Louis encephalitis, identified by neutralization with specific immune serum, was recovered from mites which had acquired the virus by feeding on a chicken previously bitten by infected mites.

As a control for these experiments and as verification of earlier results which had indicated that the colonies believed to be uninfected were still free of virus, two samples of mites from uninfected colonies were tested for virus. These two control samples consisted of 700 and 200 mites. The suspension of each sample was inoculated intracerebrally into six mice, 7 days of age. The brain of each of the twelve mice thus inoculated was passed intracerebrally to adult mice on the 12th or 13th day. None of these adult passage mice developed signs of illness.

## DISCUSSION

Hammon and his associates (7) have presented data from field and laboratory investigations which support the hypothesis that both St. Louis encephalitis and Western equine encephalomyelitis are mosquito-borne, and that the source of the infection for the mosquito is an inapparent reservoir among vertebrates, particularly the domestic fowl. However neither the virus of St. Louis encephalitis nor that of Western equine encephalomyelitis has been isolated from hibernating mosquitoes, nor is there evidence that these viruses persist in the blood of experimentally infected fowls for longer than a few days. Thus the means by which these viruses persist from year to year in an endemic area is unexplained.

Isolation of the virus of St. Louis encephalitis from chicken mites (*Dermanyssus gallinae*) in several localities in St. Louis County during a non-epidemic year (1, 2), and demonstration that the virus persists in these mites for many months by transovarian passage (3) have pointed to the possible rôle of this arachnid vector in maintaining an endemic focus in nature.

However, as previously pointed out, these observations cannot be considered significant in the epidemiology of St. Louis encephalitis unless infected mites transfer the virus to chickens by bite and unless the blood of such chickens can serve as a source of virus for blood-sucking vectors. Results described in the present report demonstrate that both naturally infected and experimentally infected chicken mites are capable of transmitting the virus of St. Louis encephalitis to normal chickens by bite. The amount of virus in the blood of chickens thus infected appears to be small since viremia was demonstrated by means of the chorioallantoic passage technique but not by direct inoculation of serum or fresh heparinized blood intracerebrally into mice. However, the amount of virus in the blood of chickens bitten by infected mites was sufficient to be acquired by uninfected chicken mites which fed upon the chicken during the period of viremia. Since one blood-sucking vector, the mite, can acquire virus from chickens fed upon by infected mites, it seems possible that other blood-sucking vectors such as the mosquito may acquire sufficient virus from such chickens to infect other animals, mammals or birds, by bite.

A complex cycle of this type may be applicable also in the epidemiology of Western equine encephalomyelitis since Sulkin (8) has succeeded in isolating the virus of Western equine encephalomyelitis from *Dermanyssus gallinae* collected in nature, and more recently Reeves *et al.* (9) have isolated the virus of Western equine encephalomyelitis from another mite, *Liponyssus sylviarum*.

## SUMMARY

Transmission of the virus of St. Louis encephalitis to normal chickens by the bite of infected mites (*Dermanyssus gallinae*) has been demonstrated. Both experimentally infected and naturally infected mites were shown to be

capable of transferring the virus of St. Louis encephalitis to chickens by bite. Virus is present in the blood of such chickens in small amounts, so that demonstration of viremia was possible only by utilizing chorioallantoic passage in hens' eggs. However, there is sufficient virus present in the blood for uninfected chicken mites to acquire the virus by feeding on chickens in which viremia has resulted from previous bite of infected mites. Thus it has been shown that the arachnid vector *Dermanyssus gallinae* is capable of transmitting the virus of St. Louis encephalitis to normal chickens by bite and that such chickens can serve as a source of virus for uninfected mites.

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# STUDIES ON THE MECHANISM OF RECOVERY IN PNEUMONIA DUE TO FRIEDLÄNDER'S BACILLUS

## I. THE PATHOGENESIS OF EXPERIMENTAL FRIEDLÄNDER'S BACILLUS PNEUMONIA\*

By LLEWELLYN SALE, JR., M.D.,† AND W. BARRY WOOD, JR., M.D.

*(From the Department of Medicine and the Oscar Johnson Institute for Medical  
Research, Washington University School of Medicine, St. Louis)*

PLATES 23 AND 24

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Recent studies on the mechanism of recovery in pneumococcal pneumonia have served to define the manner in which antibacterial chemotherapy brings about a favorable termination of the pulmonary infection (1-3). Evidence has been presented that "surface phagocytosis" is instrumental in bringing about destruction of pneumococci in the lung in the absence of type-specific immune bodies (3). Since this particular phagocytic phenomenon has not previously been described and since its general biological significance is not known, an effort has been made to determine its relationship to the mechanism of recovery in other bacterial infections, particularly those caused by encapsulated microorganisms which are naturally resistant to phagocytosis. The present investigation deals with pneumonia due to Friedländer's bacillus.

The clinical and pathological features of acute Friedländer's bacillus pneumonia have been described in detail (4-11). Although the essential characteristics of the disease are in many ways similar to those of pneumococcal pneumonia, there are two notable differences: (1) Friedländer's pneumonia causes a higher case fatality rate in human patients than does pneumococcal pneumonia; (2) Friedländer's infection of the lungs frequently leads to suppuration and the formation of lung abscess, a complication rarely encountered in pneumococcal pneumonia, except in the case of type III infection. It should be emphasized also that although Friedländer's bacillus and the pneumococcus are both encapsulated organisms possessing many characteristics in common, the former is a Gram-negative bacillus which differs in certain fundamental biological properties from Gram-positive cocci.

Although the pathologic anatomy of Friedländer's pneumonia as observed in fatal cases of the disease has been carefully described, little is known about the pathogenesis of the pulmonary lesion. Experimental Friedländer's pneumonia has been produced in dogs (12-14) and mice (15) but only the end

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† Research Fellow, Department of Medicine, Washington University School of Medicine.

stages of the resulting lesion have been studied. Since basic information is lacking concerning the pathogenesis of Friedländer's pneumonia, a study of the evolution of the pneumonic lesion has been made in white rats and is described in the present paper. Subsequent reports (16, 17) deal with (a) the action of an effective chemotherapeutic agent upon the pulmonary lesion of Friedländer's pneumonia and (b) a detailed analysis of the mechanism involved in the destruction of Friedländer's bacilli in the lung.

### *Method*

The method used to produce acute Friedländer's bacillus pneumonia in rats was essentially the same as that employed in experimental pneumococcal pneumonia. Details of the technique have been reported in a previous paper (18).

White rats, varying in weight from 175 to 300 gm. and free of chronic pulmonary infection, were inoculated intrabronchially under ether anesthesia with 0.15 ml. of a suspension of type A Friedländer's bacilli<sup>1</sup> in 6 per cent mucin. The virulence of the organism was maintained by frequent mouse passage and by storage at  $-70^{\circ}\text{C}$ . in defibrinated rabbit blood under vacuum. The number of bacilli inoculated into the left main bronchus of each rat varied from 100 to 3000. Following inoculation, each animal was kept under light ether anesthesia for 30 minutes in a vertical position to insure penetration of the mucin into the terminal bronchi. Blood cultures were taken from the tail vein at frequent intervals during the course of the infection. All surviving rats were killed with ether, and only the lungs of freshly killed animals were used for microscopic study. The lungs, after gross examination, were fixed in Zenker-formol solution by the method of Loosli (19) and sections 7 microns thick were stained by a modification of the Gram-Weigert technique (18).

### RESULTS

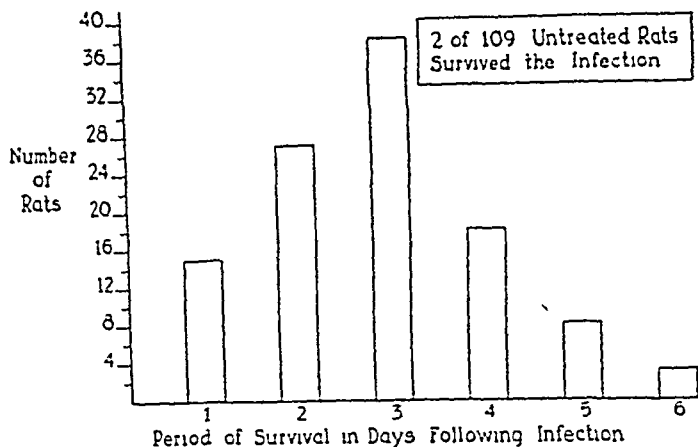
*Fatality Rate.*—The pneumonia produced by Friedländer's bacillus was fatal in all but two of 109 animals. The two surviving rats apparently developed chronic Friedländer's infection of the left lung. As is shown in Text-fig. 1, all animals of the 107 animals that died did so within 6 days, the majority succumbing on the 3rd day.

*Gross Pathology of Pulmonary Lesions.*—Representative pulmonary lesions in rats dying of Friedländer's pneumonia are diagrammed in Text-fig. 2. In over 50 per cent of the animals the lesion was confined to the left lung.

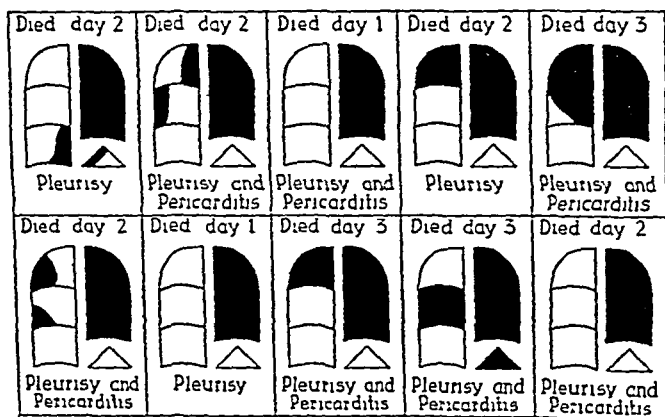
Stages in the development of the pneumonic lesion were studied in a series of twenty-six rats killed at 20 minutes, and at 2, 6, 12, 18, 24, and 36 hours after inoculation. Three to five rats were sacrificed at each interval. The lungs were examined and then fixed for histological study. The results are represented diagrammatically in Text-fig. 3.

<sup>1</sup> Strain "Chic" obtained through the courtesy of Dr. Frances Clapp of the Lederle Laboratories, Inc. Organisms from a 4 to 6 hour culture in tryptose phosphate broth were used in all experiments.

In rats killed 20 minutes after inoculation, the left lung showed a small hemorrhagic area at the site of inoculation, probably due to trauma. After 2 hours the lesion was more hyper-



TEXT-FIG. 1. Survival time of untreated rats dying of experimental Friedländer's pneumonia.










TEXT-FIG. 2. Representative pulmonary lesions in untreated rats dying of experimental Friedländer's pneumonia. All blood cultures positive at time of death.

emic and was clearly demonstrable. Rapid spread occurred after 6 hours with involvement of almost the entire left lung at 36 hours. In the first 36 hours, spread to other lobes of the lung was noted in only two of the twenty-one animals. The lesion was dark red in color during the first 24 hours, and there was little change in the size of the left lung. At 36 hours, however, the left lung was noted to be enlarged, firm, and of a gray-pink color, only the outer margin of

the lesion remaining hemorrhagic. Later lesions seen in animals dying after 48 or 72 hours were of a whitish yellow color and caused considerable increase in the size of the lung (see Fig. 1). Some lesions in animals dying after the 3rd day showed unmistakable evidence of abscess formation.

*Incidence of Bacteremia.*—The results of blood cultures taken at frequent intervals during the course of the disease are recorded in Text-fig. 3. Bacteremia did not occur during the first 6 hours of infection. After 18 hours, 36 per cent of the animals had positive blood cultures and 75 per cent were bacteremic at 36 hours. Blood cultures from all animals dying of the infection were positive.

Time after Inoculation	20 min.	2 hours	6 hours	12 hours	18 hours	24 hours	36 hours
Approximate Size of Pulmonary Lesion*							
Blood Culture Positive	0	0	0	3	7	10.	13
Blood Culture Negative	17	18	17	13	12	7	4
Incidence of Bacteremia	0%	0%	0%	18%	36%	59%	76%

\* Based on autopsy findings in 3-5 rats killed at each interval

TEXT-FIG. 3. Extent of pulmonary lesion and incidence of bacteremia at various stages of experimental Friedländer's pneumonia.

*Occurrence of Pleurisy and Pericarditis.*—Fibrinous pleurisy, pericarditis, or both, occurred constantly as complications of the fatal infection. Pleural effusion was occasionally noted as early as 6 hours and was frequent at 18 hours, particularly in the bacteremic animals. The fluid was thin and serosanguineous in rats dying in less than 18 hours and was cloudy, but rarely frankly purulent, in those surviving longer. Pericarditis was occasionally encountered at 12 hours and was frequently seen in animals dying later with bacteremia. The pericardial fluid at the time of death was purulent and occasionally of a gelatinous character. Cultures of the cloudy pleural and pericardial fluids were uniformly positive for Friedländer's bacilli.

*Histopathology of Pneumonic Lesion.*—The histological features and pathogenesis of the pneumonic lesion produced by Friedländer's bacilli were revealed in the microscopic sections cut from rats killed at the intervals designated in



Text-fig. 3 and also from rats surviving as long as 72 hours. The histology of the lesion during the first 6 hours of the infection was essentially the same as that described for experimental pneumococcal pneumonia (18). At this early stage few organisms were visible in the alveoli and the lesion contained many mucin particles, hemorrhagic edema fluid, and a light polymorphonuclear exudate. After 12 hours the full blown pneumonic lesion was easily recognized and may be briefly described as follows:—

The outer margin of the spreading lesion was characterized by an edema zone in which the alveoli were filled with fluid, essentially free of leucocytes (Fig. 2). Floating freely in the edema fluid were many bacteria which were apparently multiplying rapidly (Fig. 3). Inside this outer edema zone there appeared a zone of early consolidation in which polymorphonuclear leucocytes, together with many organisms, could be seen in the alveoli. In this area of the lesion the leucocytic exudate was thin, and very little, if any, phagocytosis was noted (Fig. 4). In the inner zone of the lesion, characterized by advanced consolidation, two contrasting processes were noted. In some areas there was marked phagocytosis, practically all of the bacteria having been taken up by the alveolar phagocytes which were still predominantly polymorphonuclear leucocytes (Fig. 5). In other adjacent areas there was apparently no phagocytosis, and the alveoli were filled with a solid mass of organisms (Fig. 6). In the spreading pneumonic lesions bacteria and edema fluid were also frequently observed in the bronchi (Fig. 7). Infected edema fluid was likewise found in alveoli adjacent to the visceral pleura, suggesting the possibility of direct penetration of the bacteria into the pleural space (Fig. 8). Only rarely were bacteria seen in the peribronchial lymphatics (Fig. 9).

In addition to the histological features noted in the early spreading lesions, there were other important microscopic findings observed in animals surviving for 48 to 72 hours. In many areas of the more advanced pulmonary lesions there were heavy deposits of fibrin in the alveoli, as shown in Fig. 10. In the central portions of the advanced lesions areas of clearing and beginning resolution were noted even in animals dying from the infection. The alveoli in such areas contained macrophages predominantly, but occasionally fibroblasts were also seen, suggesting a tendency for the lesion to become organized (Fig. 11). Finally, in the older lesions, unmistakable evidence of abscess formation was frequently noted. Microscopic examination of such abscesses revealed complete destruction of the alveolar walls and a dense confluent exudate composed largely of polymorphonuclear leucocytes (Fig. 12).

#### DISCUSSION

Experimental Friedländer's pneumonia produced in rats appears to be more acute than the natural disease in man. The essential pathological features of the experimental infection, however, are the same as those observed in fatal human cases. The lobar distribution of the lesion, the firmness and enlargement of the affected lung, the existence of abscesses in the consolidated lobes,

the large number of bacilli in many of the alveoli, the active phagocytosis by polymorphonuclear leucocytes and mononuclear cells and the frequent occurrence of bacteremia, pleurisy, and pericarditis, constitute the principal pathological findings in the experimental disease and simulate closely those noted in fatal human cases examined at autopsy (5-11).

The pathogenesis of the pulmonary lesion in experimental Friedländer's pneumonia would appear to be essentially the same as that previously described in pneumococcal pneumonia (18). The outer margin of the spreading lesion is characterized by an edema zone in which the alveoli are filled with watery edema fluid containing many bacteria. Not only do the organisms apparently multiply freely in the edema fluid but they seem to be carried mechanically by the fluid into adjacent alveoli through the pores of Cohn and the communicating alveolar ducts and bronchioles.<sup>2</sup> Early studies on the pathogenesis of pneumococcal pneumonia led to the hypothesis that the infection spread by way of the pulmonary lymphatics (21). This suggestion was later refuted by the careful experiments of Loosli (22) which conclusively proved that the spread occurred mainly by way of infected edema fluid. The rarity with which Friedländer's bacilli were found in the lymphatics in the present study would appear to indicate that the same mechanism of spread operates in both pneumococcal and Friedländer's pneumonia.

As in pneumococcal pneumonia, heavily infected edema fluid is seen in many of the large bronchi, suggesting an obvious mechanism of spread of the pneumonia to other lobes. Convincing evidence that such spread in pneumococcal pneumonia is due to infected edema fluid in the large bronchi has been presented by Robertson and Hamburger (23). The bronchial findings in the present study indicate that the same mechanism obtains in pneumonia caused by Friedländer's bacillus.

The histological features of the more central portions of Friedländer's bacillus lesions are also similar in many respects to those of the pneumococcal lesions (18). In the zone of early consolidation adjacent to the edema zone, leucocytes and bacteria are present in the alveoli, but there is little or no phagocytosis. Only in the more central zone of advanced consolidation where the alveoli are packed with leucocytes is phagocytosis noticeable. Here both polymorphonuclear leucocytes and macrophages have ingested the bacteria. That the phagocytes ingest the bacteria and ultimately destroy them, even in animals dying of fatal infections, is evidenced by the fact that in adjacent areas no bacteria remain in the alveoli and there are signs of resolution. Fibrin is prominent in the alveolar exudate of the central portion of the lesions.

Although the reaction of the murine lung to infection with Friedländer's

<sup>2</sup> The spreading process in undoubtedly accentuated by the respiratory movements of the lungs (20).

bacillus is apparently similar to its reaction to pneumococcal infection, there are several important differences in the pathogenesis of the two pneumonias. The first and most important difference concerns the number of organisms noted in the areas of consolidation. In pneumococcal pneumonia, the number of bacteria in a given alveolus is never very great, and the number appears to diminish rapidly as consolidation proceeds, so that very few organisms can be found in the central zone of advanced consolidation (18). Such rapid destruction of the offending bacteria does not always occur in Friedländer's pneumonia. Although the destructive mechanism appears to function efficiently in most parts of the lesion, in some areas Friedländer's bacilli accumulate in tremendous numbers. Here the alveoli may be filled by masses of matted organisms. The ability of Friedländer's bacillus to multiply thus freely in the face of an inflammatory exudate, distinguishes the organism from the type I pneumococcus. Since the pneumonic exudate is said to create a somewhat acid environment in the alveoli and since Friedländer's bacillus can multiply at a lower pH than pneumococcus, it is conceivable that the differences noted in the respective pneumonic lesions are due, in part at least, to differences in the cultural requirements of the two organisms.

The tendency of Friedländer's bacillus pneumonia to lead to abscess formation constitutes a second characteristic that differentiates it from pneumococcal pneumonia. Although most types of pneumococci<sup>3</sup> that invade the lung call forth a pronounced inflammatory reaction in the affected alveoli, they rarely cause destruction of either alveolar or bronchial tissues. When pneumococcal pneumonia resolves, the lung is eventually restored to normal. Friedländer's bacilli, on the other hand, not only accumulate in large numbers in certain areas of the pneumonic lesion, but destroy the alveolar walls and thus cause abscesses. In the areas of abscess formation, where there occurs a breakdown of the normal alveolar architecture, the phagocytic cells function less efficiently in destroying the invading bacteria. The cause of the decreased efficiency of the phagocytes in such areas is discussed elsewhere in connection with the mechanism of destruction of Friedländer's bacilli in the lung (17).

The third difference observed concerns the occurrence of organization in the late stages of the pneumonia. Rarely was evidence of organization noted in experimental pneumococcal pneumonia (18), whereas the fibroblastic activity characteristic of organization of the alveolar exudate was commonly encountered in Friedländer's infection. This difference can probably be attributed to a greater damage to the alveolar tissues caused by the large accumulations of Friedländer's bacilli referred to above.

<sup>3</sup> There is some evidence that pneumococcus Type III may behave more like Friedländer's bacillus in its relation to the animal host than do other types of pneumococcus (24).

## SUMMARY

Experimental pneumonia due to Friedländer's bacillus was produced in white rats by the intrabronchial inoculation of the bacilli suspended in mucin. The pneumonia was lobar in type, was almost uniformly fatal, and simulated the acute form of the natural disease in human beings.

The pathogenesis of the pneumonic lesion was studied by examination of microscopic sections of the lungs of animals killed at frequent intervals during the course of the infection. The histologic characteristics of the various stages of the pneumonia were essentially the same as those previously described in experimental pneumococcal (Type I) pneumonia except for the following differences: (1) In isolated areas of the lung in Friedländer's pneumonia many more bacteria were encountered in the alveoli than were ever noted in experimental pneumococcal pneumonia. (2) Abscess formation was common in the late stages of Friedländer's infection, whereas it was not noted in the pneumococcal lesion. (3) Organization of the alveolar exudate, rarely observed in experimental pneumococcal pneumonia, was a prominent feature of the pneumonia due to Friedländer's bacillus.

The mechanism of spread of Friedländer's lesion appeared to be the same as that of pneumococcal pneumonia. Likewise there was noted the same phagocytosis of organisms in the lungs of even bacteremic animals dying of the infection.

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## EXPLANATION OF PLATES

Microscopic sections from the lungs of rats with experimental Friedländer's bacillus pneumonia. The photomicrographs were taken by Mr. Milton K. Echtold, and all sections were stained by the Gram-Weigert technique.

## PLATE 23

FIG. 1. Gross lesion in rat dying of pneumonia 72 hours after inoculation. Complete consolidation of unilobar left lung.  $\times 2$ .

FIG. 2. Edema zone at margin of lesion. Alveoli are filled with bacteria-laden edema fluid, containing few leucocytes. Lesion is progressing from right to left. Animal sacrificed at 12 hours; no bacteremia.  $\times 200$ .

FIG. 3. Friedländer's bacilli in alveolar edema fluid at margin of lesion. Few leucocytes have yet appeared in alveoli; and the organisms appear to be multiplying freely. Animal sacrificed at 12 hours; no bacteremia.  $\times 900$ .

FIG. 4. Early consolidation showing Friedländer's bacilli and leucocytes in alveolus. Rat sacrificed at 12 hours; blood culture sterile.  $\times 625$ .

FIG. 5. Marked phagocytosis in area of consolidation. Many organisms are seen within the cells, which are predominantly polymorphonuclear. This animal had bacteremia when sacrificed at 24 hours.  $\times 1000$ .

FIG. 6. Masses of bacteria in area of advanced consolidation. No phagocytosis can be demonstrated in such areas. Rat sacrificed at 24 hours, with bacteremia.  $\times 855$ .



#### PLATE 24

FIG. 7. Edema fluid laden with Friedländer's bacilli within the lumen of a large bronchus. Rat sacrificed at 12 hours. Blood culture positive.  $\times 625$ .

FIG. 8. Edema fluid containing Friedländer's bacilli in alveoli beneath visceral pleura. Animal killed at 12 hours; no bacteremia.  $\times 850$ .

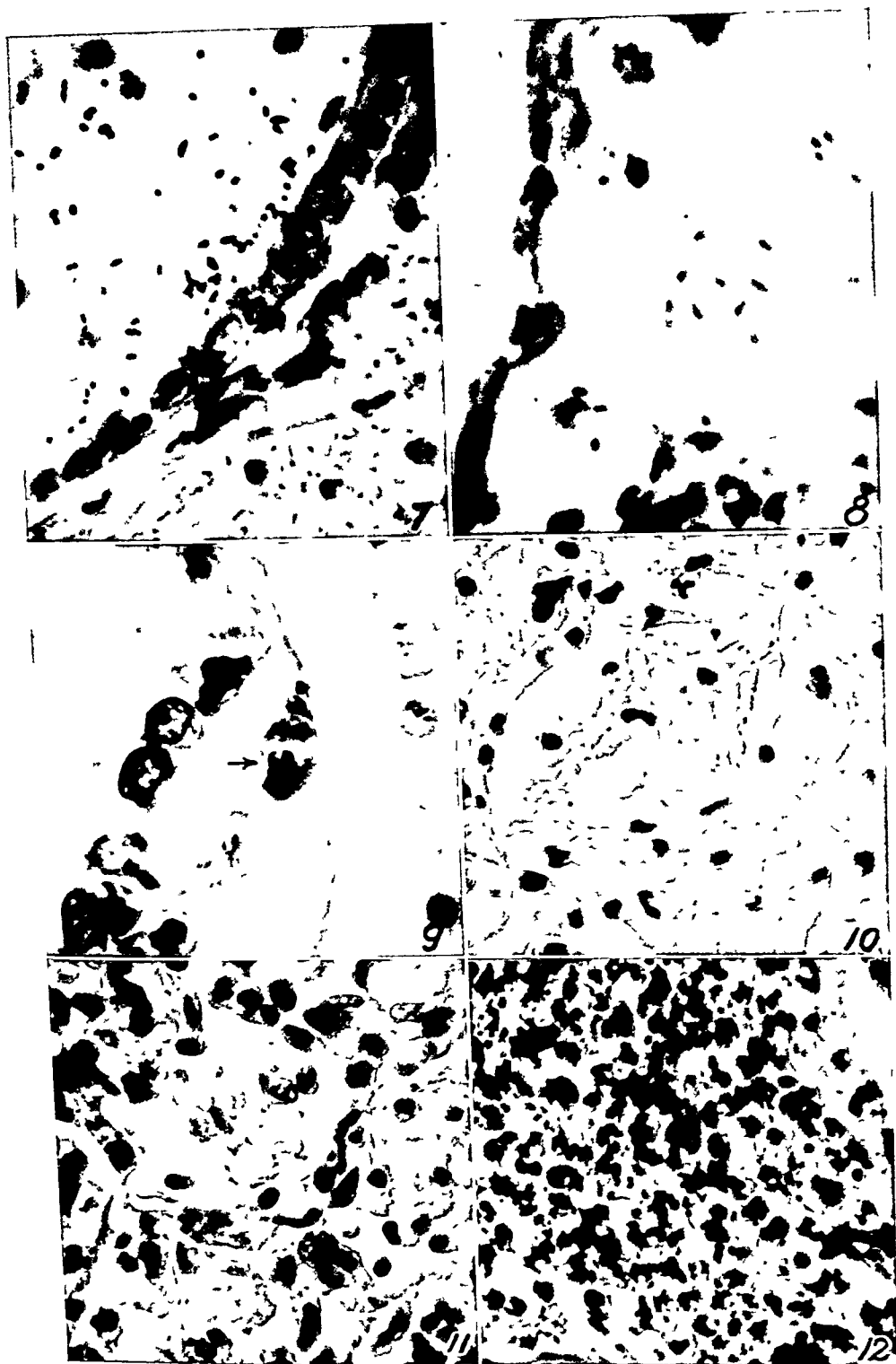
FIG. 9. Friedländer's bacilli (see arrow) in peribronchial lymphatic. Rat with bacteremia sacrificed at 24 hours.  $\times 1000$ .

FIG. 10. Fibrin deposits in alveoli in area of consolidation. Strands of fibrin can be seen passing through pores of Cohn. Rat killed at 72 hours; blood culture positive.  $\times 570$ .

FIG. 11. Clearing and early organization of lesion. Some of the cells in the alveoli are fibroblasts. Animal sacrificed at 72 hours with bacteremia.  $\times 570$ .

FIG. 12. Abscess formation in area of advanced consolidation. Leucocytes are undergoing necrosis and the alveolar walls have been destroyed. Blood culture positive after 72 hours of infection.  $\times 570$ .







# STUDIES ON THE MECHANISM OF RECOVERY IN PNEUMONIA DUE TO FRIEDLÄNDER'S BACILLUS

## II. THE EFFECT OF SULFONAMIDE CHEMOTHERAPY UPON THE PULMONARY LESION OF EXPERIMENTAL FRIEDLÄNDER'S BACILLUS PNEUMONIA\*

By LLEWELLYN SALE, JR., M.D.,† MARY RUTH SMITH,§  
AND W. BARRY WOOD, JR., M.D.

(From the Department of Medicine and the Oscar Johnson Institute for Medical Research, Washington University School of Medicine, St. Louis)

PLATES 25 AND 26

(Received for publication, May 26, 1947)

Both clinical and experimental studies indicate that infections due to Friedländer's bacillus may be favorably affected by sulfonamide chemotherapy (1-12). Little is known, however, concerning the mechanism of recovery from such infections in either human patients or laboratory animals. In the preceding paper the pathogenesis of experimental Friedländer's pneumonia in rats was described (13). The disease produced resembled acute Friedländer's pneumonia in man. The present report deals with the effect of sulfonamide chemotherapy upon the pulmonary lesion of this experimental infection.

### Methods

The detailed techniques employed in the production of experimental Friedländer's bacillus pneumonia in white rats have already been described (13). The pneumonia produced was almost uniformly fatal in untreated animals. Blood cultures were taken from the tail at frequent intervals during the course of the infection, and all lungs were fixed in Zenker-formol solution according to the method of Loosli (14). Tissue sections were stained by the Gram-Weigert technique (15).

*Sulfonamide Treatment.*—Either powdered sulfadiazine or powdered sulfamerazine<sup>1</sup> was used in all experiments. Five gm. of the powdered drug was added to 100 ml. of a 10 per cent gum acacia mixture, and appropriate amounts of the resulting suspension were introduced into the stomach through a blunt cannula by way of the mouth. Three hundred mg. of sulfadiazine (6 ml.) was given as an initial dose, at the end of 12 hours and every 24 hours thereafter. When sulfamerazine was used, 24 hour maintenance doses of 200 mg. (4 ml.) were found to be sufficient to maintain an adequate blood level. The concentrations of drugs maintained in the blood by the two dosage schedules in both normal and infected animals are charted in Text-fig. 1.

*Tests for Antibody.*—The methods used to detect circulating antibodies against Friedländer's

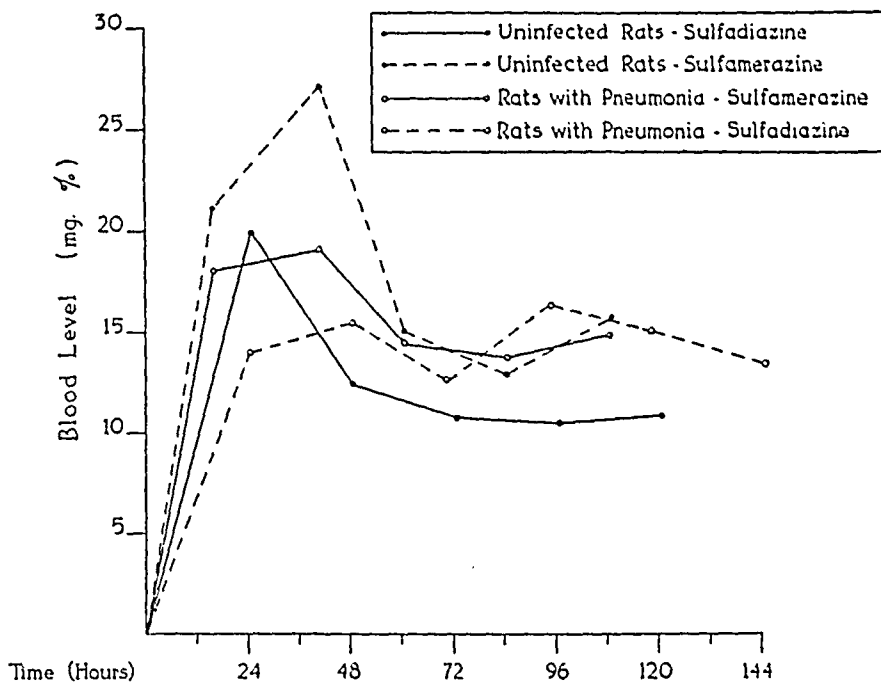
\* This study was supported by the Commonwealth Fund.

† Research Fellow, Department of Medicine, Washington University School of Medicine.

§ Recipient of Research Fellowship awarded by the Lederle Laboratories, Inc.

<sup>1</sup> Supplied through the courtesy of Dr. Benjamin Carey, Lederle Laboratories, Pearl River, New York.

bacillus included the standard mouse protection test (16) and a microscopic test for opsonins and agglutinins. In the mouse protection test 0.2 ml. of pooled serum (obtained from four to six rats) was injected intraperitoneally together with 0.2 ml. of tryptose broth containing 5 per cent para-aminobenzoic acid<sup>2</sup> and approximately 50 Friedländer's bacilli. Five mice were used in testing each lot of pooled serum, and the final results of the tests were tabulated at the end of 1 week. The procedure used in testing for opsonins and agglutinins was as follows. Washed Friedländer's bacilli obtained from 0.25 ml. of a 4 hour culture of the organisms in serum infusion broth were suspended in 0.1 ml. of the test serum, together with leucocytes obtained from peritoneal exudate of rats (17). The mixture was placed on a glass slide and incubated for 30 minutes in a sealed Petri dish lined with moist filter paper (17).



TEXT-FIG. 1. Sulfonamide blood levels in normal rats and rats with experimental pneumonia treated by mouth with sulfamerazine and sulfadiazine. Each point on the curves represents the average of values obtained on five or more animals. (For dosage, see text.)

At the end of the period of incubation smears were made and stained with methylene blue. Both phagocytosis and agglutination of extracellular organisms were considered in recording the final results of the tests on each lot of serum.

## RESULTS

*Effect of Sulfonamide Chemotherapy upon Fatality Rate.*—When treatment with either sulfadiazine or sulfamerazine<sup>3</sup> was begun 6 hours after inoculation more than 90 per cent of the animals survived the infection. As shown in

<sup>2</sup> To neutralize the bacteriostatic effect of the sulfonamide contained in the rat serum.

<sup>3</sup> At no time was any appreciable difference noted in the effect of sulfadiazine and sulfamerazine upon the experimental infection.

Table I, there were two deaths from pneumonia among the 33 rats so treated. More than half of these animals were allowed to survive longer than 10 days before being sacrificed. When treatment was withheld until 9 hours after inoculation, the survival rate was appreciably lower, and after 12 hours only half of the animals survived the pneumonia. Treatment begun 18 hours after inoculation appeared to have no appreciable effect upon the course of the infection. In all subsequent treatment experiments sulfonamide therapy was started 6 hours after inoculation.

*Effect of Sulfonamide Chemotherapy upon the Gross Pneumonic Lesion.*—The action of sulfonamide chemotherapy upon the pulmonary lesion was studied in a series of treated rats sacrificed at 6, 18, 24, 36, 42, 66, 90, and 168 hours after the start of treatment. Three rats were killed at each interval, and the lungs were examined in the gross before being fixed for histological study, (Text-fig. 2).

TABLE I  
*Survival Rate among Rats with Experimental Friedländer's Pneumonia Treated with Sulfonamide at Various Intervals after Inoculation*

Time after inoculation that treatment was begun	No. of rats treated	No. of rats surviving pneumonia	Survival
<i>hrs.</i>			<i>per cent</i>
6	33	31	94
9	19	14	73
12	10	6	60
18	9	0	0








Six hours after the start of treatment the gross lesion occupied about half the left lung and was bright red in color. The border between the normal and consolidated lung was hemorrhagic and irregular, presenting the same appearance as in untreated animals 12 hours after inoculation (13). Both the size of the lesion and the character of its border indicated that the drug had exerted little or no effect upon the spreading pneumonia during the first 6 hours of treatment. At 18 hours the lesion was slightly larger, but its margin was now sharp and lacked the irregular hemorrhagic appearance noted at 6 hours. Thereafter there occurred no further spread of the pneumonia, and at 24, 36, and 42 hours the lesion became grayer and more firm in consistency. After 66 and 90 hours of treatment the area of consolidation appeared to be slightly smaller than at 18 hours, and definite contraction of the hard gray lesion was noted at the end of 1 week. At each of the above intervals the site of the original mucin inoculation was identifiable as a dark red area which differed in color from the pneumonic lesion and failed to spread.

In marked contrast to the pathological findings in untreated animals (13) neither pleurisy nor pericarditis was noted in rats sacrificed during sulfonamide therapy.

*Effect of Chemotherapy upon Bacteremia.*—Frequent blood cultures were taken during the course of treatment; the results are recorded in Text-fig. 2. After 6 hours of treatment bacteremia was present in more than 50 per cent of the

animals. At no later time, however, did the incidence exceed 15 per cent, and after 1 week's treatment all blood cultures were sterile.

*Histopathology of Pulmonary Lesion in Treated Animals.*—Tissue sections were studied from the lungs of all of the rats sacrificed at the intervals stated in Text-fig. 2. Additional rats were also killed at 3, 18, and 24 hours after the start of treatment, and the lungs of three animals at each interval were examined histologically to determine the effect of treatment upon the lesion.

Time after Treatment	6 hours	18 hours	42 hours	66 hours	90 hours	1 week	Untreated Rats**
Approximate Size of Pulmonary Lesion*							
Blood Culture Positive	7	2	0	2	1	0	5
Blood Culture Negative	6	11	13	12	14	13	0
Incidence of Bacteremia	54%	15%	0%	14%	7%	0%	100%

\* Based on autopsy findings in 3 rats killed at each interval

\*\* Untreated rats died in less than 100 hours

TEXT-FIG. 2. Effect of sulfonamide chemotherapy upon pulmonary lesion and incidence of bacteremia in experimental Friedländer's pneumonia. Treatment begun 6 hours after inoculation.

As shown in Fig. 1, the morphology of the Friedländer bacilli in the outer edema zone at the end of 3 hours of therapy appeared to be normal. Examination of the rest of the lesion revealed it to be identical with the early pneumonia observed in untreated animals. After 6 hours of treatment, the edema zone was still prominent (Fig. 2), but the Friedländer bacilli in the edema-filled alveoli were now noted to be enlarged, pleomorphic, and irregularly stained (Fig. 3), indicating bacteriostasis (18). At 18 hours the edema zone was no longer present at the margin of the lesion (Fig. 4), and the pneumonic process appeared to have stopped spreading. In the outermost alveoli, where the bacteria were most plentiful, at both 18 and 24 hours, organisms could be seen in the cytoplasm of the polymorphonuclear leucocytes (Fig. 5). After 36 hours of treatment phagocytosis of the bacteria appeared to be more complete (Fig. 6), and at later intervals large mononuclear cells could be seen to be taking part in the phagocytic process (Fig. 7). At the end of 90 hours the alveolar exudate was predominantly mononuclear; extracellular bacteria were now no longer visible and most of the intracellular organisms appeared to have been digested by the phagocytes (Fig. 8). Signs of clearing of the pneumonic process were prominent at the end of the 7 day period of treatment.

*The Effect of Sulfonamide Therapy upon the Formation of Abscesses in the Lung.*—The lungs of a few of the treated rats exhibited definite lung abscesses

at the time of autopsy. The incidence of abscess formation was higher in animals treated 12 hours after inoculation than in those treated at 6 hours. In one experiment treatment was begun 6 hours after inoculation and was maintained for a period of 2 weeks. Two rats so treated and sacrificed on the 35th day had small but definite lung abscesses from which Friedländer's bacilli were recovered in pure culture.

*The Relation of Phagocytosis in the Lung to the Presence of Circulating Antibody.*—The serum from sulfonamide-treated rats recovering from Friedländer's bacillus pneumonia was tested for mouse-protective, opsonizing, and agglutinating antibodies at the end of the 1st, 2nd, 3rd, 4th, and 7th days of treatment. Although phagocytosis was prominent in the lungs within the first 24 hours of treatment, no antibody could be detected in the blood serum until after 4 or more days of treatment (see Table II).

TABLE II

*Tests for Antibody in Serum of Rats with Experimental Friedländer's Pneumonia Treated with Sulfonamide*

Duration of pneumonia	Mouse protection test*	Opsonocytaphagic test	Agglutination test
<i>hrs.</i>			
24	0-5	0	0
48	1-5	0	0
72	0-5	0	0
96	0-5	±	0
168	3-5	+	+

\* First figure indicates number of mice surviving at end of 1 week; second figure refers to number of mice inoculated.

## DISCUSSION

Friedländer's bacillus pneumonia induced in white rats by the methods described in the present studies (and not treated) constitutes an acute pulmonary infection which is almost uniformly fatal. Although somewhat more fulminating in its course than human Friedländer's pneumonia, the experimental murine infection resembles very closely the acute form of the human disease (13). The experiments here reported show that the experimental pneumonia in rats, in spite of its fulminant character, can be controlled by adequate sulfonamide therapy provided the drug is administered early in the disease. The manner in which the drug exerts its beneficial effect is revealed by the histology of the recovery process in the lung.

In the untreated animal Friedländer's bacilli appear to cause consolidation of the lung by the same mechanism as does the pneumococcus (15). The organisms can be seen in large numbers in the outer edema zone of the lesion where they are carried mechanically into adjacent alveoli by the edema fluid, thus

producing a spread of the lesion. As in experimental pneumococcal pneumonia (19) sulfonamide chemotherapy appears to exert its main effect in this spreading edema zone. Six hours after the start of treatment the Friedländer bacilli in the edema-filled alveoli exhibit morphological changes indicative of bacteriostasis (18), and shortly thereafter the pneumonic lesion ceases to spread. The appearance of edema fluid in the outermost portion of the spreading lesion constitutes the earliest stage of inflammation in the infected alveoli. When the lesion stops spreading as the result of bacteriostasis, phagocytes accumulate in all of the infected alveoli that previously contained only edema fluid, and since no further spread of the infection occurs, the edema zone disappears from the margin of the lesion. Once phagocytes have accumulated in sufficient number in the infected alveoli, they destroy the bacilli by the same phagocytic process that operates in the zone of consolidation in untreated animals (13). The sulfonamide drug brings about recovery by inhibiting the growth of the bacteria at the advancing margin of the lesion, thereby enabling the alveolar phagocytes to destroy the bacteria that have invaded the lung. Only in areas of abscess formation do the phagocytes fail to destroy all of the offending organisms.

Although from these studies little doubt appears to exist as to the manner in which sulfonamide chemotherapy causes recovery in Friedländer's pneumonia, the exact mechanism involved in the phagocytosis and ultimate destruction of the bacteria remains obscure. Friedländer's bacillus, like pneumococcus, possesses a protective capsule which renders the organism resistant to phagocytosis. Why then should phagocytosis occur in the pneumonic lesions of both treated and untreated animals? In the case of experimental pneumococcal pneumonia (18), phagocytosis is observed in the first 24 hours of the infection and occurs even in the lungs of bacteremic animals. Thus it appears unlikely that circulating antibody is responsible for the phagocytic reaction. Likewise, in the present experiments, no antibody could be detected in the blood of rats recovering from Friedländer's pneumonia until several days after phagocytosis had begun in the lungs. All of the evidence thus far advanced indicates that the ultimate recovery of animals with Friedländer's pneumonia when given sulfonamide depends upon the phagocytosis of the offending bacteria, and yet no satisfactory explanation can be given for the occurrence of phagocytosis of the encapsulated organisms in the absence of opsonins. Therefore, further experiments have been carried out to elucidate the exact mechanism of this non-antibody phagocytic reaction in the lung, and to determine the reason for its failure to operate efficiently in areas of abscess formation. The results of these experiments are reported in the following paper.

#### SUMMARY

Sulfonamide chemotherapy was found to cure rats of an otherwise fatal form of experimental Friedländer's bacillus pneumonia when treatment was begun 6



hours after inoculation. Most of the pneumonic lesions cleared completely, but an occasional animal exhibited small residual abscesses in the previously consolidated lung.

The recovery process taking place in the lungs was studied histologically at various intervals during therapy. As in the case of pneumococcal pneumonia, the principal action of the sulfonamide was upon the bacteria in the advancing edema zone at the periphery of the pneumonic lesion. The bacteriostatic action of the drug appeared to stop the spread of the pneumonia, and the Friedländer bacilli were ultimately ingested and destroyed by the phagocytic cells in the alveolar exudate.

The phagocytosis of bacteria in the lung was shown to be unrelated to the presence of antibody in the blood.

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## EXPLANATION OF PLATES

Tissue sections from the lungs of rats with experimental Friedländer's bacillus pneumonia treated with sulfonamide. Fig. 5 was photographed by Mr. Cramer Lewis; the remaining figures by Mr. Milton K. Echtold. All sections were stained by the Gram-Weigert technique.

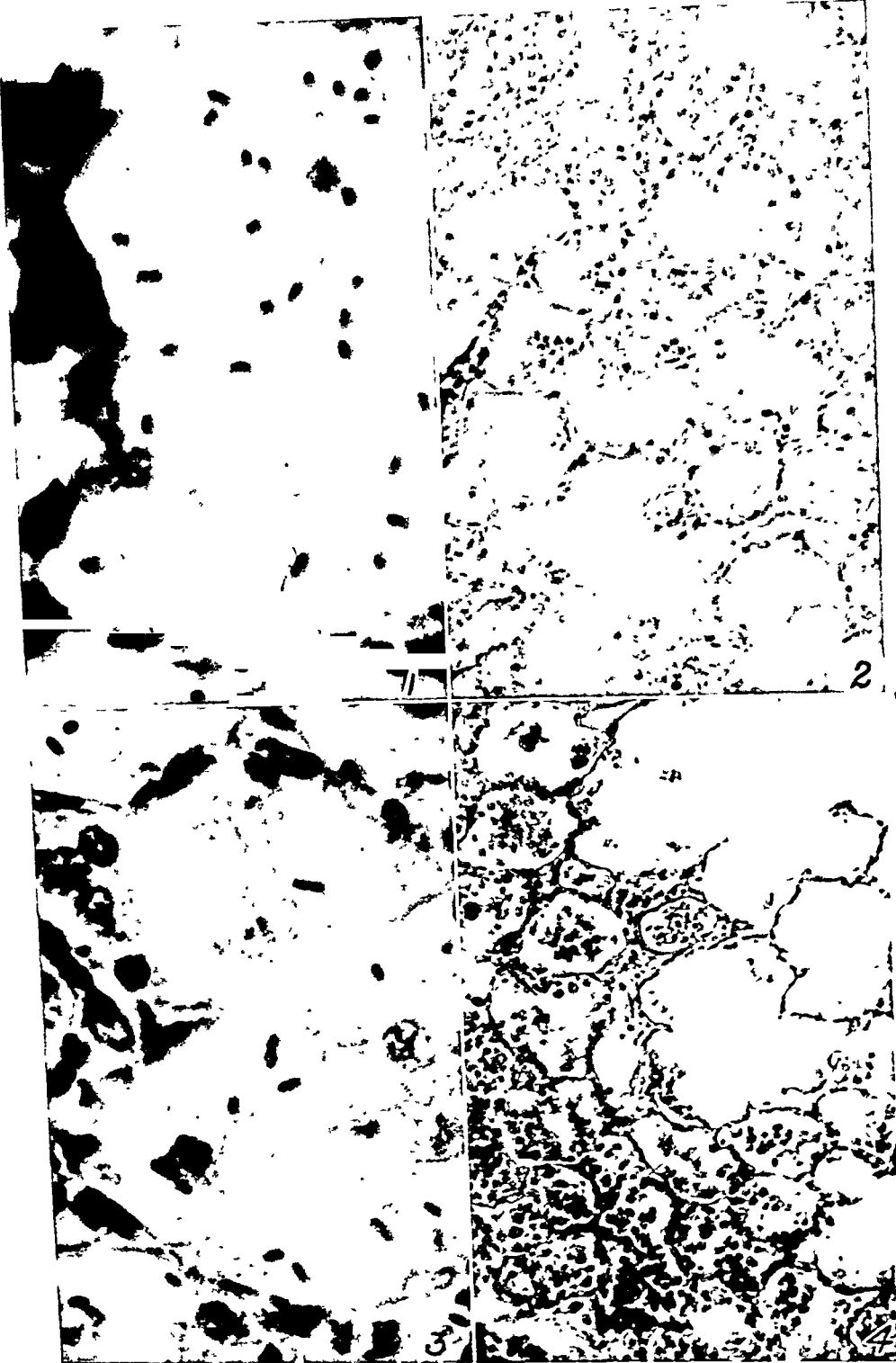
## PLATE 25

FIG. 1. Bacteria-laden edema fluid in alveolus at margin of spreading lesion 3 hours after the start of treatment. The bacteria as yet exhibit none of the characteristic morphological signs of bacteriostasis.  $\times 1500$ .

FIG. 2. Edema zone still present at margin of lesion 6 hours after the start of treatment.  $\times 200$ .

FIG. 3. Pleomorphism of Friedländer's bacilli, indicating early bacteriostatic effect after 6 hours of treatment.  $\times 1500$ .

FIG. 4. Disappearance of edema zone from the margin of lesion 18 hours after beginning of treatment.  $\times 200$ .



(Sale *et al* : Mechanism of recovery in pneumonia. II)

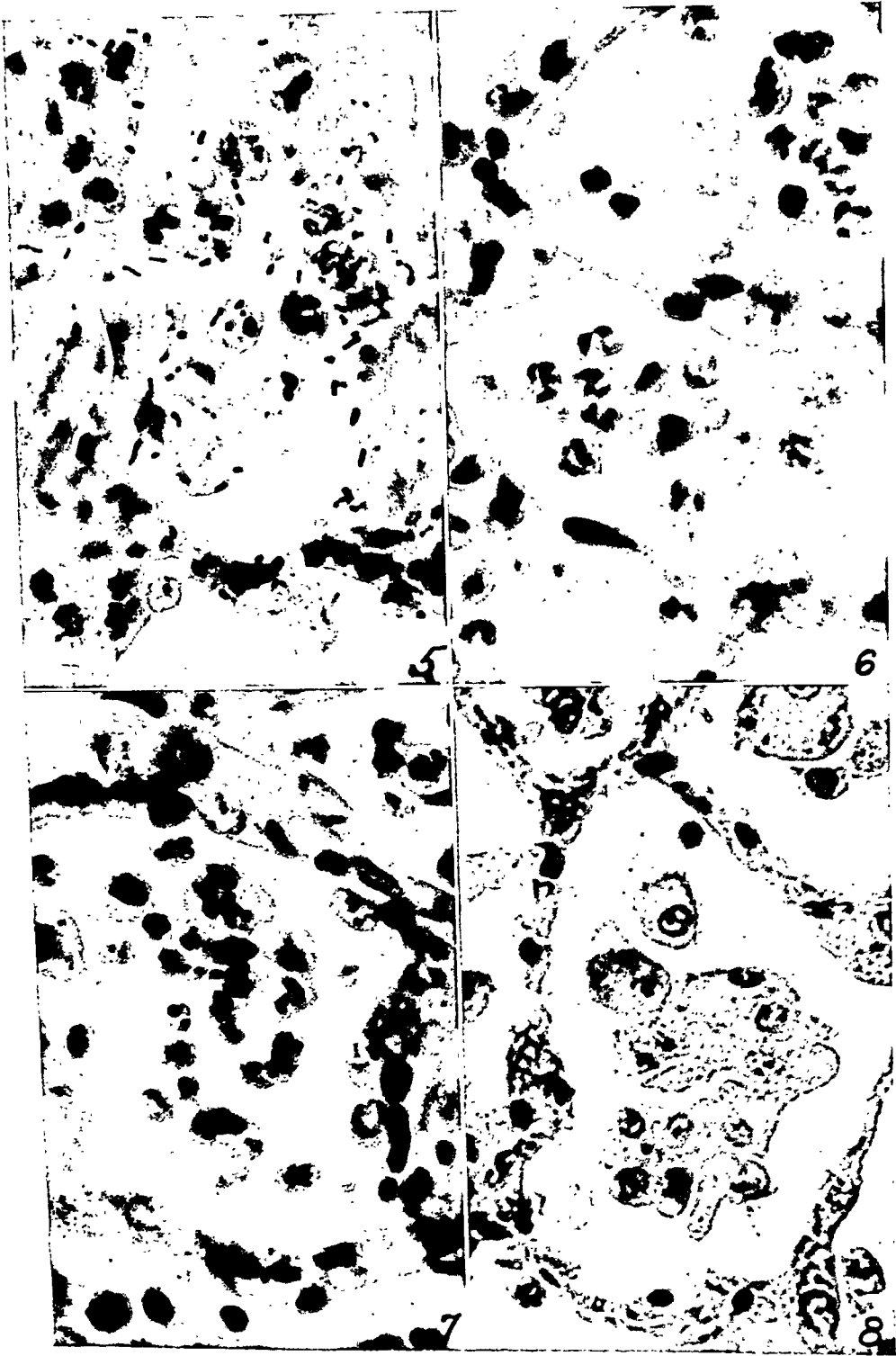
PLATE 26

FIG. 5. Early phagocytosis of Friedländer's bacilli in rat sacrificed at end of 24 hours of treatment.  $\times 1500$ .

FIG. 6. More complete phagocytosis of Friedländer's bacilli by leucocytes in alveolar exudate after 36 hours of treatment.  $\times 500$ .

FIG. 7. Macrophage reaction with a few intracellular organisms. This animal was sacrificed 66 hours after the start of treatment; the macrophage is the predominant cell.  $\times 855$ .

FIG. 8. Lesion after 1 week of treatment showing marked clearing with only macrophages in the alveoli. Bacteria are no longer visible in the cytoplasm of the phagocytes.  $\times 1000$ .



(Sale *et al.*: Mechanism of recovery in pneumonia. II)



# STUDIES ON THE MECHANISM OF RECOVERY IN PNEUMONIA DUE TO FRIEDLÄNDER'S BACILLUS

## III. THE RÔLE OF "SURFACE PHAGOCYTOSIS" IN THE DESTRUCTION OF THE MICROORGANISMS IN THE LUNG\*

By MARY RUTH SMITH,† and W. BARRY WOOD, JR., M.D.

(From the Department of Medicine and the Oscar Johnson Institute for Medical Research, Washington University School of Medicine, St. Louis)

PLATES 27 AND 28

(Received for publication, May 26, 1947)

Evidence has been presented that encapsulated Friedländer's bacilli are destroyed by leucocytes in the pneumonic lungs of rats treated with sulfonamides, even when the animals possess no detectable opsonins in the blood (1). Similar results were reported previously in a study of experimental pneumococcal pneumonia (2, 3) and the destruction of organisms in the absence of antibody was shown to be due to a physical phenomenon designated "surface phagocytosis" (4). The experiments reported in the present paper demonstrate that under similar conditions the same surface mechanism is responsible for the phagocytosis of Friedländer's bacilli and results in rapid killing of the organisms.

### (A) *The Resistance of Encapsulated Friedländer's Bacilli to Phagocytosis in a Fluid Medium*

Friedländer's bacillus in its virulent form, like pneumococcus, possesses a capsule which protects it against phagocytic cells. When suspended in a fluid medium with active leucocytes, Friedländer's bacilli are not phagocytosed unless previously coated with specific opsonin. The ability of the fully encapsulated organism to resist phagocytosis in a fluid medium is shown by the following experiment.

*Experiment 1.*—Friedländer's bacilli from 2 ml. of a 4 hour culture in tryptose phosphate broth were washed twice with iced gelatin-Locke's solution and suspended with washed leucocytes harvested from the peritoneal cavities of four rats injected intraperitoneally 24 hours previously with starch-aleuronat (4). The cells were washed from the peritoneal cavity of each rat with cold gelatin-Locke's solution containing liquaemin<sup>1</sup> (1:1000 concentration) and after centrifugation were rewashed at least once before being added to the bacteria. All supernatant fluid was poured off the final centrifugates of the bacterial and leucocytic suspensions before they were mixed. The final cell-bacillus mixture contained approximately ten phagocytes and twenty-five bacilli per oil immersion field when placed beneath a

\* This study was supported by the Commonwealth Fund.

† Recipient of Research Fellowship awarded by the Lederle Laboratories, Inc.

<sup>1</sup> A commercial preparation of the sodium salt of heparin—1 ml. = 10 mg. (Roche Organon, Inc.)

coverslip. Such mixtures, incubated at 37°C. in hanging drop preparations or between coverslips, uniformly failed to show appreciable phagocytosis (Fig. 1). Direct observation of the phagocytes in the hanging drop mixtures<sup>2</sup> revealed that bacilli were frequently pushed aside by advancing leucocytes and were not phagocyted in spite of repeated contacts with the cells (Figs. 2 to 5).

*(B) Demonstration that Phagocytosis of Friedländer's Bacilli Occurs in the Lung in the Absence of Local as Well as Circulating Antibody*

Although phagocytosis of Friedländer's bacilli occurs in the lungs of rats whose serum contains no detectable opsonin (1), it may be argued that the observed phagocytic reaction is due to the local accumulation of antibody in the pulmonary tissues. The result of the following experiment would appear to rule out this possibility.

*Experiment 2.*—Normal rat lungs were fixed for 24 hours in 10 per cent formalin to eliminate all living cells and unprecipitated proteins. The fixed lungs were washed for at least 5 days in tap water and were rinsed with gelatin-Locke's solution before being used. Into the left main bronchus of each formalin-fixed lung was injected a suspension of leucocytes and Friedländer's bacilli in gelatin-Locke's solution. The bacteria used in the suspension were obtained by centrifugation from 3.5 ml. of a 4 hour culture in tryptose phosphate broth containing 1 per cent rabbit serum. The harvested organisms were washed twice in cold gelatin-Locke's solution before being added to washed leucocytes obtained from the peritoneal cavities of six rats (see above). To insure penetration of the leucocyte-bacillus mixture into the alveoli, each lung was expanded gently under negative pressure at the time the mixture was introduced by cannula into the bronchus. The injected lungs were incubated for 2 hours at 37°C. and then fixed in Zenker-formol solution, sectioned, and stained by the Gram-Weigert technique (5).

As shown in Fig. 6 phagocytosis of the Friedländer bacilli occurred even in the formalin-fixed lungs, which contained no possible source of opsonin other than the phagocytic cells themselves. It can be concluded, therefore, that the ability of leucocytes to attack virulent Friedländer's bacilli in the lung is not dependent upon the accumulation of local antibody in the pulmonary tissue.

*(C) Phagocytosis of Friedländer's Bacilli on Tissue and Other Surfaces*

From the foregoing observations it appeared likely that the non-antibody phagocytosis of Friedländer's bacilli is due to the same type of surface mechanism that has been shown to operate in the case of pneumococci (4). To test this hypothesis experiments were performed in which mixtures of leucocytes and Friedländer's bacilli were placed on various solid surfaces and incubated in a moist atmosphere at 37°C. for 60 minutes. At the end of incubation impression

<sup>2</sup> Unstained organisms from 4 hour cultures in tryptose phosphate broth were used in all experiments except when preparations were made for photography, in which case the bacteria were stained with carbol-fuchsin as previously described (4). Repeated control experiments demonstrated that the stained organisms behaved toward the leucocytes exactly as did the unstained organisms.



smears were made from the test surfaces and were stained with methylene blue. The exact techniques employed in carrying out the experiments have already been described (4).

As shown in Table I, leucocytes having access to the surfaces of normal or boiled tissues, or to such rough inert material as moistened filter paper are able to phagocytose the encapsulated Friedländer bacilli in the absence of antibody (Fig. 7). No such phagocytosis occurs on the smooth surface of a glass coverslip<sup>3</sup> (Fig. 1). Leucocytes suspended in the fluid medium of a hanging or suspended drop (4) likewise fail to phagocytose the organisms. Thus, fully encapsulated Friedländer's bacilli, like pneumococci, are phagocytosed in the absence of opsonins only when the leucocytes have access to a suitable surface upon which to operate.

TABLE I

*Tests for Phagocytosis on the Surfaces of Fresh Rat Tissues, Boiled Rat Tissues,\* Moistened Filter Paper, and on Glass*

Lung.....	+
Liver.....	+
Spleen.....	+
Boiled lung.....	+
Boiled liver.....	+
Boiled spleen.....	+
Filter paper.....	+
Glass coverslip.....	-

\* Boiled for 30 minutes.

#### (D) Direct Visualization of Surface Phagocytosis

The technique employed to visualize directly the surface phagocytosis of Friedländer's bacilli was similar to that used in the study of pneumococcal infections (4).

Normal rat lungs, fixed for 24 hours in 10 per cent formalin, were washed for several days in tap water, embedded in paraffin, and cut in sections of 5 microns thickness. The cut sections were floated onto "O" coverslips and allowed to dry before being washed in xylol to remove the paraffin. The mounted tissues were finally run through alcohol, washed repeatedly in distilled water, and allowed to dry. A small drop of bacillus-leucocyte mixture in gelatin-Locke's solution was placed on each mounted lung section, which was then inverted as a hanging drop preparation in the cavity of a hollow ground slide and rimmed with vaseline to prevent drying. Each preparation was placed in the warm stage of the microscope (37 C.) and was observed under the oil immersion lens. The phagocytic process could thus be watched directly in the alveoli close to the margin of the drop where the layer of fluid was sufficiently thin to confine the leucocytes and the bacteria to the alveolar cavities.

<sup>3</sup> If the leucocyte-bacillus mixture is sufficiently concentrated, some phagocytosis will occur on the glass surface due at least in part, to "intercellular surface phagocytosis." (See below.)

As is illustrated by the photographs in Figs. 8-15, phagocytosis of the Friedländer bacilli resulted when the organisms were pinned against the alveolar walls by the migrating leucocytes. It should be emphasized that the leucocytes appeared to wander about purely at random, there being no evidence of either positive or negative chemotaxis. The sequences photographed were selected to illustrate the phagocytic process, but frequently the leucocytes singled out for observation moved about the alveolus without trapping any of the bacteria against the alveolar walls. In such cases no phagocytosis resulted. Occasionally bacteria were caught between the surfaces of two or more leucocytes that happened to collide with one another. Bacteria so trapped between the surfaces of contiguous cells were often phagocyted. This type of phagocytosis referred to as "inter-cellular surface phagocytosis," will be described in more detail elsewhere (6).

*(E) Evidence that Surface Phagocytosis Brings about Destruction  
of Friedländer's Bacilli*

Apparent intracellular digestion of Friedländer's bacilli following phagocytosis by the surface mechanism was demonstrated as follows:—

Leucocyte-Friedländer's bacillus mixtures prepared as described above (section A) were incubated on the surface of moistened filter paper for 45 minutes (see section C) and were then washed from the filter paper with 10 ml. of iced gelatin-Locke's solution. The cells, many of which contained intracellular organisms, were twice centrifuged in iced gelatin-Locke's solution at 800 R.P.M. for 5 minutes to eliminate extracellular bacilli. The centrifuged cells were finally suspended in approximately 10 volumes of normal rat serum containing liquaemin in a concentration of 1:100 and were incubated at 37°C. The final suspensions were made in normal rat serum in order to provide an optimal environment for the leucocytes. (It should be reemphasized that normal rat serum contains no detectable antibody against this strain of Friedländer's bacillus (1).) Smears of the incubating cell suspension were made every 15 to 30 minutes and were stained with methylene blue. The numbers of intracellular and extracellular bacilli noted per 500 polymorphonuclear leucocytes are recorded in Table II.

The results of the bacterial counts indicate a pronounced decrease in the number of intracellular organisms during the period of incubation following phagocytosis. The decrease in intracellular organisms appears to be due to digestion of the phagocytosed bacteria. However, because of the concomitant increase in the number of extracellular bacilli during incubation, it is possible that the decrease in intracellular organisms was due merely to the escape of bacteria from the cytoplasm of the phagocytes rather than to digestion. A method was devised, therefore, to observe directly the fate of the phagocytosed bacteria during the period of incubation.

As in the preceding experiment phagocytosis was brought about on moistened filter paper, and leucocytes containing phagocytosed Friedländer's bacilli were suspended in normal rat serum after the extracellular organisms had been removed by centrifugation. A drop of the serum-cell mixture was spread, in as thin a layer as possible, on a mounted section of fixed rat

lung (see section D) and was observed in the warm stage of the microscope under oil immersion lens.

In each experiment a single selected leucocyte containing two or more phagocytized bacilli was watched for several hours. Repeatedly the outlines of the bacilli were seen to become faint as if from partial digestion, and occasionally the organisms disappeared completely from the cytoplasm of the leucocyte. Only once was intracellular multiplication of the bacilli observed, and in this instance the leucocyte when first observed was non-motile and apparently dead.

Because of these last observations additional experiments were carried out to test further the ability of phagocytized organisms to multiply in the cytoplasm of the cells.

TABLE II

*Data Indicating Intracellular Digestion of Friedländer's Bacilli Following Surface Phagocytosis*

Time of incubation (37°C.) following surface phagocytosis	No. of intracellular bacilli per 500 leucocytes	No. of extracellular bacilli per 500 leucocytes
<i>min.</i>		
0	755	102
45	632	341
90	468	> 1000
150	356	> 1000

Leucocytes that had phagocytized Friedländer's bacilli on moistened filter paper, and from which most of the extracellular organisms had been removed by centrifugation were smeared on a glass slide and stained with methylene blue. A direct count on the stained preparation was made to determine the number of leucocytes containing Friedländer's bacilli. A second portion of the centrifuged cells was spread gently over the surface of a thin coverslip and before the preparation could dry, the coverslip was inverted onto a warm glass slide on the surface of which had been placed a drop of melted agar (45°C.) containing trypan blue. The trypan blue-agar mixture was made by mixing equal parts of 3 per cent agar (in double strength tryptose phosphate broth) and 1 per cent trypan blue in Locke's solution. Repeated control experiments showed that this concentration of trypan blue in the agar exerted no demonstrable effect upon the growth of unphagocytized Friedländer's bacilli. When the inverted coverslip was placed upon the drop of trypan blue-agar, capillary action caused the agar to spread out rapidly between the two glass surfaces, embedding the cells in the upper plane just beneath the coverslip. After the agar had solidified, the coverslip was rimmed with paraffin, and the preparation was incubated at 37°C. for 2 hours before being examined under the microscope.

In repeated experiments only about 10 per cent of the leucocytes containing Friedländer's bacilli exhibited colonies of bacteria,<sup>4</sup> and most of the intracellular colonies were observed to be in leucocytes, the nuclei of which were stained with trypan blue. Since trypan blue, in the concentrations employed, stains the nuclei of dead cells and is not taken up by living cells (7), it was concluded that

<sup>4</sup> These data were later confirmed by using the more direct agar-slide method. (See below.)

intracellular multiplication occurred primarily in the cytoplasm of non-viable leucocytes.

The foregoing results indicated that phagocytosed Friedländer's bacilli were eventually killed by the leucocytes, provided the latter were still viable. Since the process of digestion appeared to be relatively slow, sometimes lasting for several hours, a technique was devised to determine the approximate speed with which the phagocytosed bacilli were *killed* by the leucocytes. The method used was based upon a test for the viability of organisms previously exposed to the intracellular environment of phagocytic cells.

Concentrated suspensions of Friedländer's bacilli and rat leucocytes in gelatin-Locke's solution containing 10 per cent rat serum<sup>5</sup> were incubated for 30 minutes on moistened filter paper as in previous experiments. The leucocytes were then washed from the surface of the paper with iced gelatin-Locke's solution and were rewashed three times to remove extracellular bacteria.<sup>6</sup> The final centrifugation was carried out in cold tryptose phosphate broth, rather than Locke's solution, and the centrifugate was transferred to an iced mortar containing 0.25 gm. of sterile sand. The sand-cell mixture was ground for 15 seconds with a mortar to break up the leucocytes and free the phagocytosed bacteria. The ground mixture was taken up in 5 ml. of cold tryptose broth, and the sand and cellular debris were separated from the bacteria by slow centrifugation. The bacilli remaining in the supernatant were finally concentrated by centrifugation in the cold (20 minutes, at 2000 R.P.M.), and the supernatant was discarded. The centrifuged bacteria thus freed from the leucocytes, were finally suspended in 0.5 ml. of 2 per cent liquid agar (45°C.) and were incubated between two thin layers of solid agar prepared as follows:—

Melted 2 per cent agar at 45°C. was smeared onto the clean surface of a coverslip in a very thin layer. This first layer of agar was allowed to solidify but not dry. A drop of the agar containing the bacteria was then spread in a similar manner over the first layer and was allowed to solidify. A third thin layer of plain agar was immediately spread over the surface of the second, and as soon as the agar had solidified, the preparation was incubated for 2 hours in a sealed Petri dish lined with moistened filter paper. Upon being removed from the Petri dish, the agar surface was stained for 1 minute with methylene blue and was washed in tap water. The wet preparation was finally inverted, mounted on a glass slide, and rimmed with melted paraffin to prevent drying of the agar. Ready diffusion of the methylene blue through the top layer of agar caused clear staining of the bacteria in the middle agar layer. It was found necessary to use three layers of agar, as described, in order to prevent disruption of colonies during the staining procedure.

In such agar-slide preparations each viable organism was easily identified since it formed a definite colony in the solid medium (Fig. 16). Dead organisms, on the other hand, remained as single isolated bacilli (Fig. 17). When non-phagocytosed Friedländer's bacilli<sup>7</sup> were tested by the same method, 99 to 100 per cent of them multiplied to form colonies. In contrast, more than one-third of the phagocytosed bacteria freed from the cytoplasm of the leucocytes remained as single organisms in the agar. Their failure to multiply indicated that they had been killed by the leucocytes apparently during the 30 minute period of incubation on filter paper. Ex-

<sup>5</sup> To preserve maximum activity of leucocytes (4).

<sup>6</sup> Cells separated from bacteria by slow centrifugation (800 R.P.M., for 5 minutes).

<sup>7</sup> The usual proportion of phagocytes to bacteria was included in these control mixtures, but no phagocytosis was allowed to take place, since the incubation was carried out on glass slides rather than on filter paper. The control mixtures were ground with sand in the same manner as the test mixtures.

posure of Friedländer's bacilli at 37°C. to the intracellular environment for periods longer than 30 minutes (up to 2 hours) did not materially influence the results of the experiment.

Although the rapid bactericidal action of surface phagocytosis was thus clearly demonstrated, it should be emphasized that the agar-slide method, as used in these experiments, was relatively crude and did not yield quantitatively accurate data. All errors involved, however, were such as to minimize rather than exaggerate the actual bactericidal effect of the phagocytosis.<sup>8</sup>

#### DISCUSSION

Surface phagocytosis has been shown to be of prime importance in the mechanism of recovery in pneumococcal pneumonia since it brings about destruction of pneumococci in the lung in the absence of antibody. In the present studies of experimental Friedländer's bacillus pneumonia phagocytosis of encapsulated bacilli has likewise been observed in the lung in the absence of both circulating and local opsonins. Analysis of the non-antibody phagocytic reaction has revealed that the same surface factors are involved as in the case of pneumococcus. Only by pinning the encapsulated Friedländer bacilli against the alveolar or bronchial surfaces, or against the surfaces of adjacent phagocytes, can the leucocytes engulf the unopsonized organisms. The bacilli floating freely in a fluid medium are only pushed about by the leucocytes and are not taken into the cells.

Since Friedländer's organism exerts no apparent chemotactic effect upon the leucocytes, the surface mechanism of phagocytosis would be relatively inefficient in ridding the lung of bacteria were it not for the extremely dense population of leucocytes which eventually accumulates in each infected alveolus.<sup>9</sup> When the spread of the pneumonia has been stopped by chemotherapy (1), the crowding of phagocytic cells into the infected alveolar spaces and bronchi finally results in all of the bacteria being pinned against tissue surfaces or trapped between two or more viable leucocytes. Thus all of the bacteria remaining in the lung may in time be phagocytosed by the surface mechanism.

Once the bacilli enter the cytoplasm of the phagocyte they are rapidly killed. Although digestion of the phagocytosed Friedländer organisms appears to be slower than that of pneumococci and may not be complete for several hours,

<sup>8</sup> The principal sources of error in the method were as follows: (1) Killed organisms that had been partially digested were often difficult to identify as bacilli and therefore were not included in the final counts. (2) The relatively large number of viable bacteria released from each of the dead leucocytes in which intracellular multiplication occurred, tended to distort the final count in favor of the living organisms. (3) All bacilli that remained outside of the leucocytes in the filter paper preparations and were not removed by the differential centrifugation appeared in the final counts as viable bacilli. (4) Whereas, occasional single bacilli (killed) may have been missed in the counting process, no viable organisms could have been overlooked because of the obvious colonies formed.

<sup>9</sup> The density of the leucocytic population in typical lesions of bacterial pneumonia is apparent from routine tissue sections taken from areas of advanced consolidation (5, 9).

many of the bacilli are rendered non-viable in less than 30 minutes. Intracellular multiplication occurs only rarely and appears to result from premature death of the phagocyte. Even under the conditions of *in vitro* experiments approximately 90 per cent of the phagocytes survived long enough to kill all phagocytosed organisms and thus prevent intracellular multiplication. Since surface phagocytosis of Friedländer's bacilli can be demonstrated in the lung, and since the ingested bacilli are rapidly killed by the leucocytes, it is only logical to conclude that this non-antibody form of phagocytosis plays just as important a part in the mechanism of recovery in Friedländer's pneumonia as it does in pneumococcal pneumonia.

It is not implied by the foregoing statement that all Friedländer's bacilli that invade the lung are destroyed by surface phagocytosis. Some of the bacilli are carried away from the alveoli and bronchi by lymphatic drainage, and some eventually enter the blood stream (9). How those organisms that escape from the lung are ultimately destroyed remains to be determined. It can be safely said, however, that of those Friedländer's bacilli that remain in the lung, the great majority appear to be killed by the surface mechanism of phagocytosis.

As has already been pointed out (1), pneumonia caused by Friedländer's bacillus differs from Type I pneumococcal pneumonia in one important respect. Whereas Type I pneumococcus rarely causes permanent damage to pulmonary tissue, the Friedländer bacillus not infrequently multiplies to such an extent in certain alveoli that necrosis results and a lung abscess is formed. In such abscessed areas the phagocytes are unable to destroy all of the bacilli, even when the animal is treated intensively for many days with sulfonamide (1). The failure of the leucocytes to rid abscesses of bacteria would appear to be due to at least two factors. First, the absence of the normal alveolar walls in the abscessed areas deprives the leucocytes of much of the tissue surface upon which they operate in the intact lung. Thus surface phagocytosis is rendered relatively inefficient. Secondly, most of the leucocytes, particularly in the center of a lung abscess are either non-viable or so sluggish that they cannot attack the bacteria. Leucocytes deprived of oxygen soon become non-motile and lose their phagocytic properties (8). Since the principal source of oxygen for leucocytes in a lung abscess is the intact alveolar capillaries at the periphery of the lesion, it is not surprising that the phagocytes in the central portion of the abscess fail to destroy the bacteria. These same two factors would appear to account at least in part for the failure of systemic chemotherapy to cure empyema and other pyogenic complications of pneumococcal as well as Friedländer's pneumonia (4).

#### SUMMARY

Phagocytosis of encapsulated Friedländer's bacilli has been demonstrated in the lungs of rats in the absence of both circulating and local antibody. The

mechanism of phagocytosis independent of antibody has been shown to be due to the same surface factors that operate in the phagocytosis of Type I pneumococcus under similar conditions. Direct observation of the phagocytic process reveals that leucocytes in the lung can phagocyte unopsonized Friedländer's bacilli only by trapping them against the surfaces of alveolar walls or bronchi, or by pinning them against the surfaces of adjacent leucocytes. Evidence is presented that Friedländer's bacilli thus phagocytosed are rapidly killed in the cytoplasm of the phagocytic cells. Reasons are discussed for the failure of prolonged chemotherapy to cure lung abscesses that not infrequently complicate the pneumonia due to Friedländer's bacillus.

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## EXPLANATION OF PLATES

Fixed smears and agar-slides stained with methylene blue (Figs. 1, 7, 16, and 17), lung section stained by the Gram-Weigert technique (Fig. 6), and bacteria in vital preparations stained with carbolfuchsin (Figs. 2 to 5 and 8 to 15). Fixed smears, agar-slides, and lung section photographed by Mr. Cramer Lewis.

## PLATE 27

FIG. 1. Failure of leucocytes to phagocyte Friedländer's bacilli on surface of glass slide. Although in intimate contact with the cells the bacilli have not been phagocytosed.  $\times 1800$ .

FIGS. 2 to 5. Series of photomicrographs showing failure of leucocyte to phagocyte Friedländer's bacilli in hanging drop.

FIG. 2. Advancing pseudopods of cell are just coming into contact with two clumps of bacilli. Time, 1:05½ p.m.  $\times 1250$ .

FIG. 3. Advancing cell has pushed clumps of bacilli apart and is in intimate contact with the bacteria. Time, 1:06 p.m.  $\times 1250$ .

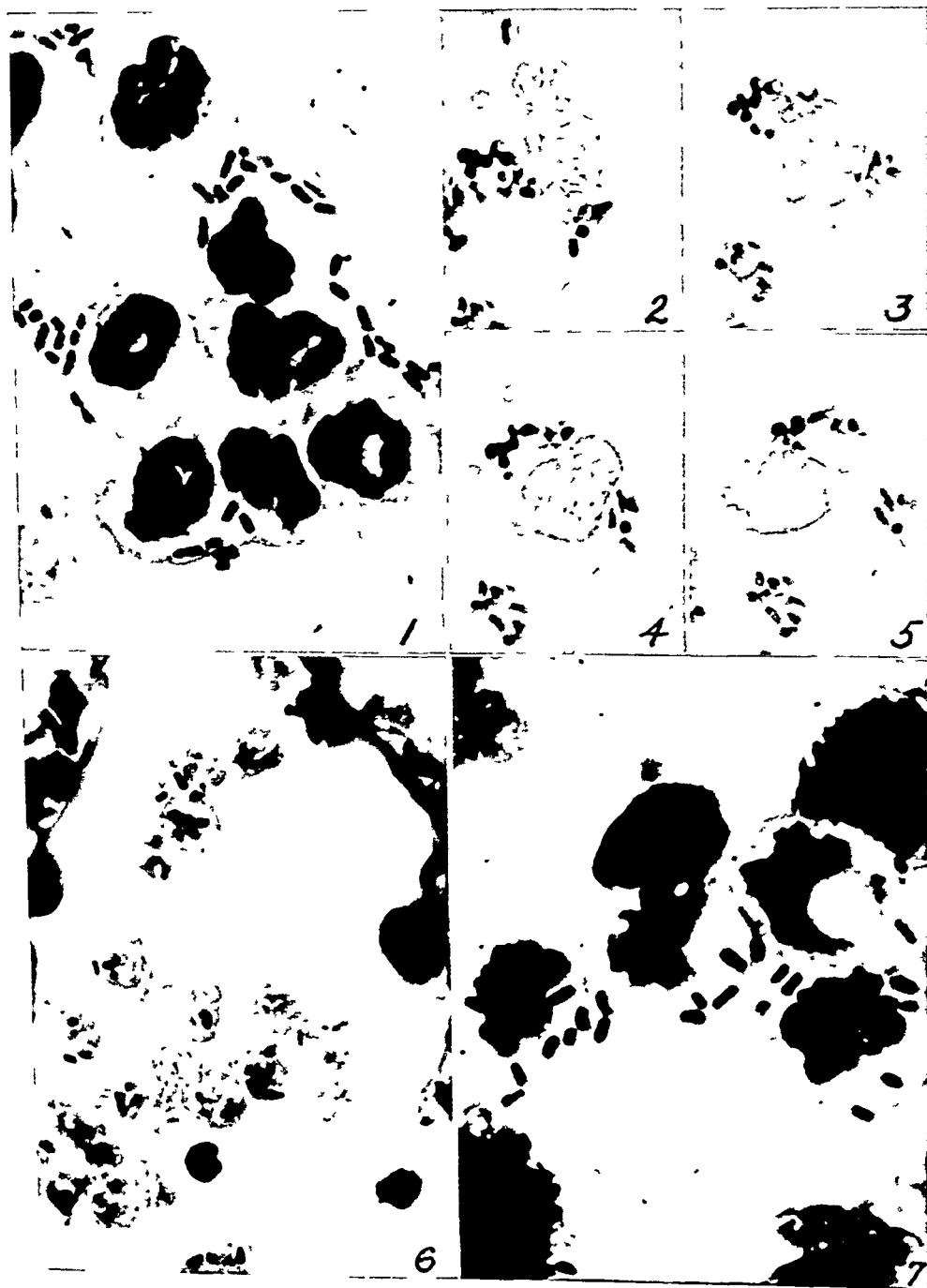
FIG. 4. Cell passes between the two clumps of bacilli. Time, 1:06½ p.m.  $\times 1250$ .

FIG. 5. Cell continues on its way leaving bacilli behind without having phagocytosed any of them. Time, 1:07 p.m.  $\times 1250$ .

FIG. 6. Phagocytosis of Friedländer's bacilli following injection of bacillus-leucocyte mixture into formalin-fixed rat lung.  $\times 1300$ .

FIG. 7. Phagocytosis of Friedländer's bacilli on surface of moistened filter paper.  $\times 1800$ .





(Smith and Wood Mechanism of recovery in pneumonia. III)

## PLATE 28

FIGS. 8 to 12. Series showing surface phagocytosis in section of normal rat lung.

FIG. 8. Leucocyte is seen approaching Friedländer's bacilli near alveolar wall. Time, 12:30 p.m.  $\times 1250$ .

FIG. 9. Leucocyte has reached alveolar wall and is about to trap bacilli against the tissue surface. Time, 12:31 p.m.  $\times 1250$ .

FIG. 10. Cell has trapped some of the bacilli against the wall and is in process of phagocytizing them. Time, 12:32 p.m.  $\times 1250$ .

FIG. 11. Having phagocytized several of the bacilli, the leucocyte is moving along the alveolar wall. Time, 12:35 p.m.  $\times 1250$ .

FIG. 12. The cell continues to migrate up the alveolar wall; the bacilli can still be seen clearly in the cytoplasm of the leucocyte. Time, 12:40 p.m.  $\times 1250$ .

FIGS. 13 to 15. Series showing surface phagocytosis of Friedländer's bacilli in another section of rat lung.

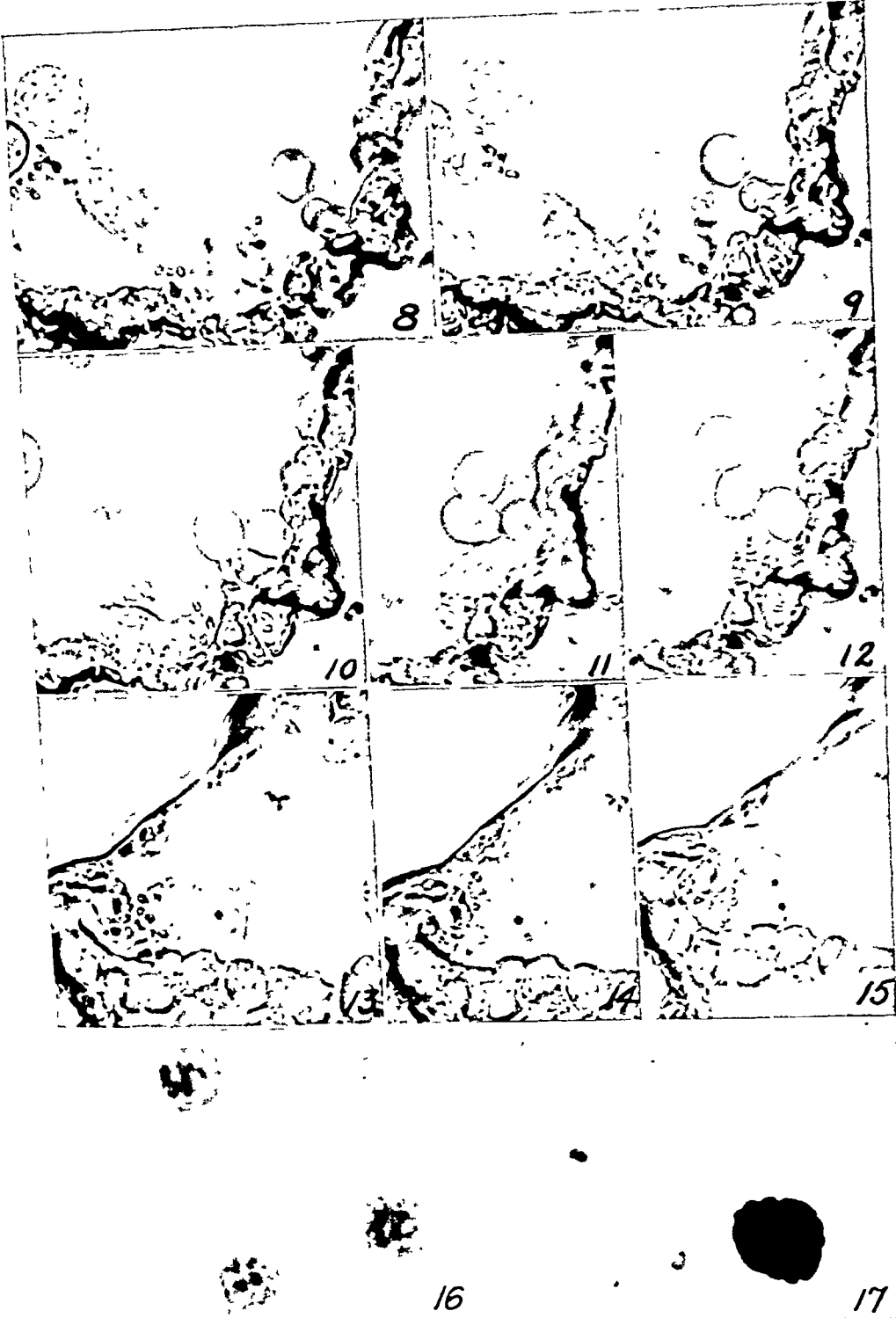
FIG. 13. Leucocyte is just making contact with bacilli trapped in corner of alveolus. Time, 12:35 p.m.  $\times 1250$ .

FIG. 14. Leucocyte has pinned bacilli against alveolar wall and is phagocytizing them. Some of the bacilli can already be seen in the phagocyte. Time, 12:37 p.m.  $\times 1250$ .

FIG. 15. Phagocytosis complete with most of bacilli in cytoplasm of the leucocyte. Time, 12:40 p.m.  $\times 1250$ .

FIG. 16. Multiplication of non-phagocytized Friedländer's bacilli in agar slide. Colony formation indicates that each of the three bacilli that were originally in the field was viable.  $\times 1800$ .

FIG. 17. Failure of three out of four previously phagocytized Friedländer's bacilli to multiply in agar slide. The three single bacilli that failed to multiply in the agar had been killed by leucocytes, whereas the viable organism formed a typical colony. The bacillus nearest to the colony is partially obscured by an overlying bit of cellular debris.  $\times 1800$ .





# STUDIES ON THE CONGLUTINATION REACTION, WITH SPECIAL REFERENCE TO THE NATURE OF CONGLUTININ

BY ALEXANDER S. WIENER, M.D., JANE G. HURST,  
AND EVE B. SONN-GORDON

*(From the Serological Laboratory of the Office of the Chief Medical Examiner of  
the City of New York)*

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For a long time no satisfactory explanation could be found for the observation that many Rh-negative patients with clinical evidence of Rh sensitization had no demonstrable Rh antibodies in their sera. Recent work (1, 2) has shown that the reason for the apparent absence of Rh antibodies from the sera of such patients is that individuals sensitized to any antigen may form either or both of two major varieties of specific antibodies, namely, agglutinins (bivalent antibodies) and blocking antibodies or glutinins (univalent antibodies). Rh blocking antibodies are characterized by their inability to clump Rh-positive red cells in saline media, though they are specifically adsorbed by such cells and render the cells inagglutinable by subsequently added specific anti-Rh agglutinating sera. According to Wiener (3, 4) plasma (and serum) contains a substance called conglutinin, apparently a colloidal aggregate of plasma proteins and probably identical with the so called X-protein, which is adsorbed by red cells after they have been specifically sensitized by univalent antibodies, thus causing them to stick together (conglutination).

Briefly, therefore, serological clumping may be brought about by two distinct mechanisms, agglutination and conglutination, which have the following distinguishing features. Agglutination occurs in saline media and is not improved by the presence of plasma or serum; according to the present authors' views this reaction takes place in a single stage through the action of bivalent (or multivalent) antibodies which "link" the multivalent red cells together. Conglutination, on the other hand, occurs only in the presence of plasma or serum or other media containing conglutinin. This reaction takes place in two stages: first the specific combination between red cells and the corresponding univalent antibodies, and then adsorption of the non-specific conglutinin by the sensitized red cells causing the cells to "stick" together. Thus, conglutinin plays an analogous rôle in the conglutination reaction to that of complement in serological hemolysis. Evidence has been obtained that individuals sensitized to the A and B factors can similarly form these two varieties of specific antibodies

(5, 6) and there is no reason to doubt the general immunological significance of the phenomenon (7).

From the foregoing it seems apparent that the intensity of the clumping in the conglutination test will depend not only on the titer of blocking antibody (or glutinin) but also on the quantity and quality of the conglutinin. The use of a potent conglutinin should increase the sensitivity of the conglutination test in much the same way that a potent complement reduces the amount of sheep cell amboceptor required to cause specific hemolysis of sheep erythrocytes. It therefore seemed worth while to investigate what factors influence the quality of conglutinin, and the purpose of this paper is to present the results of some of these experiments.

### *Materials and Methods*

To facilitate the comparison of results obtained on different days the Rh antisera used in the investigations to be described were obtained from only two individuals. Neither of the sera was capable of clumping Rh-positive red cells in saline media but strong clumping occurred in the presence of plasma. All the tests were done by the conglutination technic, that is, by titrations carried out in the following way (4). A series of progressively doubled saline dilutions of the Rh antiserum was first accurately prepared, and one drop of each dilution was transferred to a corresponding series of empty tubes (inside diameter 7 mm.). To each tube was then added a drop of a fresh 2 per cent saline suspension of Rh-positive red blood cells, and the rack containing the tubes was shaken and placed in a water bath at body temperature. After 1 hour the tubes were centrifuged at low speed and the supernatant fluid removed *as completely as possible* with the aid of a fine capillary pipette. (It is important that this step be carefully done because the presence of any residual fluid vitiates the results.) One drop of the solution being tested for its conglutinin content was then added to each tube. The red cells were resuspended by vigorously shaking the tubes and the mixtures incubated for another hour in the water bath. The sediments at the bottom of each tube were then dislodged by gently shaking the tubes, though more vigorously than in the case of Rh agglutination tests, and the reactions were read microscopically by placing the tubes on the stage of a microscope under the low power objective.

For those unfamiliar with the conglutination technic it may be mentioned that mistakes in reading the reactions can be avoided if the following points are borne in mind:

In contrast to the reading in the agglutination test, the naked eye reading of the pattern of the sediment is of no value in the conglutination test, because in the presence of concentrated plasma negatively reacting bloods do not give the typical negative sediment reading. One must rely entirely on the microscopic reading. Also centrifugation does not improve the reactions and should be avoided. In the presence of plasma, centrifugation tends to pack the cells together into rounded masses, and when the tubes are subjected to the same amount of shaking as is necessary to render the negative control homogenous, weak reactions may be destroyed. Accordingly, for the conglutination test sedimentation is preferable to centrifugation.

The plasma used in the experiments was obtained by collecting blood in tubes containing dried oxalate powder, prepared as for the Wintrobe-Landsberg sedimentation test. The tubes were inverted several times until the powder was completely dissolved and the plasma was separated either by sedimentation or by centrifugation. To obtain serum, blood was collected in dry clean tubes and allowed to clot. The clots were rimmed and the serum was

separated either by centrifugation or by allowing the clots to retract. The serum and plasma were usually used fresh, or stored in the refrigerator for not longer than 2 or 3 days.

The 25 per cent human albumin solution used in the experiments was purchased from the Cutter Laboratories; the 30 per cent bovine albumin solution from Armour and Company. The concentrated immune human gamma globulin used in our experiments was the preparation distributed by Cutter Laboratories for measles and pertussis prophylaxis.

All titrations were performed at least in duplicate against Rh-positive group O cells of types Rh<sub>1</sub> and Rh<sub>2</sub>, and control tests with type rh cells were always included. In general, the results were the same regardless of the type of the Rh-positive cells. All of the experiments were repeated several times, but in the tables only a few representative protocols will be given.

## RESULTS

The experiments to determine the nature of congenitinin will be presented under three headings, namely, (1) studies on oxalated plasma and serum (natural congenitinin), (2) studies on the effect of the addition of purified albumin and globulin to plasma (fortified congenitinin), and (3) studies on purified solutions of albumin and globulin and mixtures of these solutions (synthetic congenitinin).

### *Studies on Oxalated Plasma and Serum*

Pooled oxalated plasma from a number of normal adults was mixed in different proportions with saline solution in order to determine the effect of dilution on the congenitinating action of plasma. The following series of dilutions were prepared: 9 parts of plasma and 1 part of saline; 8 parts of plasma and 2 parts of saline; 7 parts of plasma and 3 parts of saline, etc., and then the congenitinating activities of these dilutions were compared quantitatively with that of the original plasma. As shown in Table I, the activity progressively diminished as more and more saline was introduced, and no reactions were obtained when the concentration of the plasma was lower than 40 per cent of its original strength. When equal parts of plasma and saline were mixed, the titer obtained was only one-tenth as high as that obtained with the original plasma and the reactions were much less distinct.

This explains why the sera from certain Rh-negative patients with clinical evidence of Rh sensitization, when titrated by the agglutination method, that is, in saline media, often show distinct clumping in the first tube of the titration but little or none in any of the higher dilutions. The clumping observed in the first tube in such cases is not due to agglutination but to congenitination, as can be confirmed by repeating the titration, using the congenitination technic. In the first tube of a titration, the serum being tested is diluted only by the equal volume of the red cell suspension, so that, as shown in Table I, it is still possible for congenitination to occur owing to the action of residual congenitinin in the patient's own serum. These findings also explain the conflicting reports of British (8) and American workers (9, 10) concerning the frequency with which

anti-Rh agglutinins can be detected in the sera of sensitized patients. The technic used by the British workers (8) apparently permitted conglutination to occur and hence their reports that almost 100 per cent of sensitized Rh-negative individuals have detectable anti-Rh "agglutinins" in their sera are due to the failure to distinguish between clumping due to agglutination and clumping due to conglutination. American workers on the other hand, found that less than 50 per cent of sensitized patients have anti-Rh agglutinins in their sera, because the technic used by these workers precluded the occurrence of conglutination. Confusion can be avoided if the terms "agglutinin" and "bivalent antibody" are reserved for those antibodies which produce clumping in saline

TABLE I  
*Effect of Dilution of Plasma on its Conglutinin Content*

Proportion of saline and plasma in mixture tested		Dilution of anti-Rh serum (patient R.)								Titer*
Pooled plasma	Saline	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
10	0	++±	++±	++±	++±	++	++	±	—	96
9	1	++±	++±	++	++	±±	+	—		64
8	2	++	++±	++	++	±±	±	—		48
7	3	++	++	++	++	±±	±	—		48
6	4	++	++	±±	±±	±	—			24
5	5	+	+	+	±	—				10
4	6	±	—							1
3	7	—	—							0

\* The titer is the reciprocal of the highest dilution giving a one plus reaction. Where no tube showed a one plus reaction, the titer is estimated by interpolation.

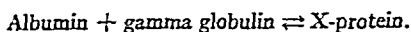
media (agglutination) while the terms "blocking antibody," "glutinin," or "univalent antibody" are employed for those antibodies which can produce clumping only in the presence of plasma proteins (conglutination). As an all inclusive term "Rh antibodies" should be used instead of "Rh agglutinins," in order to avoid ambiguity. Similarly, when describing the reactions, "clumping" should be used as the all inclusive term because the use of the term "agglutination" would cause confusion.

The observations shown in Table I are readily explained if, as has been suggested in a previous paper (3), conglutinin is identical with the so called X-protein studied by Pedersen (11). As Pedersen has pointed out, in dilute solutions plasma and serum behave in the ultracentrifuge as if they contain two major protein components, albumin and gamma globulin. As the dilution is decreased a third component (beta globulin or X-protein) appears which thereafter increases linearly with increase in total protein concentration, at the



expense of the albumin and gamma globulin. Pedersen concludes that X-protein is a reversibly dissociable compound formed by albumin, globulin, and certain lipids, having a total particle weight of about a million. He also believes that X-protein probably contains 3 or 4 times as many molecules of albumin as of globulin. As shown in the diagram in Pedersen's monograph, X-protein was first detectable in the ultracentrifuge when there was a total protein concentration of about 1.5 gm. per 100 cc., while in our experiments the lowest protein concentration to give a positive reaction was about 3 per cent. This indicates that the minimal concentration of X-protein to produce a distinct reaction in the conglutination test is about  $\frac{3}{4}$  to 1 gm. per 100 cc.

From the nature of X-protein it seems obvious that it would be impossible to obtain X-protein in the form of a pure solution. The formation of X-protein can be expressed by the following equilibrium equation:



In view of a recent report by Jakobowicz and Bryce (12), the experiment shown in Table I was repeated, using cerebrospinal fluid as a diluent instead of saline solution, and identical results were obtained. The phenomenon observed by these authors must therefore be ascribed to the water content and not any special property peculiar to cerebrospinal fluid. The bizarre results obtained by them were probably due to the use of sera containing a mixture of agglutinating and blocking antibodies of approximately equal titers. In our hands, such sera gave erratic results in tests performed in saline media. Thus, in some experiments strong clumping was obtained, while in tests made on other days with serum of the same derivation, the clumping was weak or absent, owing to competition between the two sorts of antibodies present in the serum.

In order to determine the effect of heating on conglutinin, samples of pooled plasma and serum were heated for one-half hour at 56°C. in the water bath. The conglutinating activities of the heated and unheated plasma and serum were then compared. As shown in Table II, in accordance with findings previously reported (4), pooled unheated serum is distinctly less active than pooled unheated plasma. Heating improves the activity of serum but weakens the activity of plasma so that the two approximate each other. The deleterious effect on plasma is readily explained because heating causes the fibrinogen to precipitate out and thus converts the plasma to serum. Aside from precipitating fibrinogen, heating at 56°C. does not appear to damage conglutinin, but instead seems to stabilize it, judging from the effect of heating on serum. This would seem to make the use of heated serum for conglutination tests desirable, except that heated serum has a greater tendency to produce rouleaux formation than either fresh serum or fresh plasma.

As has just been pointed out, plasma is much more active than serum as a source of conglutinin. Since the only known substance present in plasma that

is lacking from serum is fibrinogen, the increased activity must be attributed to this substance. When one considers the low concentration of fibrinogen as compared with the other plasma proteins it may seem surprising that its presence should cause such a marked increase in the conglutinating activity. This finding will be more acceptable if one considers that fibrinogen in conjunction with other proteins makes up the colloidal complex conglutinin, and that it is the quality and quantity of this conglutinin, not merely the concentration of proteins in solution, which determine the conglutinating activity. In the studies

TABLE II  
*Effect of Heating\* on the Conglutinin Content of Pooled Human Plasma and Human Serum*

Material tested	Test cells	Dilution of anti-Rh serum (patient R.)								Titer	Average titer
		Un-diluted	1:2	1:4	1:8	1:16	1:32	1:64	1:128		
Pooled adult plasma (unheated)	Rh <sub>1</sub>	+++	+++	+++	+++	+++	++	±	—	48	48
	Rh <sub>2</sub>	+++	+++	+++	+++	+++	++	±	—	48	
	rh	—	—	—	—	—	—	—	—	—	
Pooled adult serum (unheated)	Rh <sub>1</sub>	++	++	++	++	±	—	—	—	12	18
	Rh <sub>2</sub>	++	++	++	++	±	±	—	—	24	
	rh	—	—	—	—	—	—	—	—	—	
Pooled adult plasma (heated)	Rh <sub>1</sub>	++	++	++	++	±	±	—	—	24	36
	Rh <sub>2</sub>	+++	+++	+++	++	++	±	±	—	48	
	rh	—	—	—	—	—	—	—	—	—	
Pooled adult serum (heated)	Rh <sub>1</sub>	++	++	++	++	+	±	—	—	16	32
	Rh <sub>2</sub>	++	++	+++	+++	++	++	—	—	48	
	rh	—	—	—	—	—	—	—	—	—	

\* The heated specimens were kept in a water bath at 56°C. for one-half hour.

of Pedersen discussed above the fibrinogen content of the serum was not taken into account.

The conglutinating activities of samples of plasma of different normal individuals and patients were next compared in order to determine whether there are individual variations in the conglutinin content of plasma from normal individuals, and whether the conglutinin content is affected by particular diseases. This work is still in progress and can be reported only in a preliminary manner at the present time. No significant difference could be demonstrated in the conglutinin content of plasma from different normal individuals or from the same individual at different times. A small but significant increase in the conglutinating activity was demonstrated in some patients with pneu-

monia and other infectious diseases, which is readily understandable in view of the high fibrinogen content of plasma of such patients, as demonstrated also by the high sedimentation rate. That increased fibrinogen was not always the explanation for the increased conglutinin content of plasma was indicated by experiments on the corresponding blood serums in which an increased conglutinin content could also at times be demonstrated. Other important factors to be taken into consideration are the albumin-globulin ratio and the state of hydration of the individual.

TABLE III

*Comparison of Conglutinin Content of Adult Plasma, Adult Serum, Fetal Plasma, and Fetal Serum*

Material tested	Test cells	Dilution of anti-Rh serum (patient R.)									Titer	Average titer
		Undiluted	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256		
Pooled adult plasma	Rh <sub>1</sub>	++	++	++	++	++	±	±	—	—	48	72
	Rh <sub>2</sub>	++±	++±	+++	++±	++	++	±	±	—	96	
	rh	—	—	—	—	—	—	—	—	—	—	
Pooled adult serum	Rh <sub>1</sub>	±	±	++	++	±	±	—	—	—	24	32
	Rh <sub>2</sub>	++	++	++	++	++	±	—	—	—	40	
	rh	—	—	—	—	—	—	—	—	—	—	
Pooled fetal plasma	Rh <sub>1</sub>	±	±	±	±	±	—	—	—	—	20	22
	Rh <sub>2</sub>	±	±	++	++	±	±	—	—	—	24	
	rh	—	—	—	—	—	—	—	—	—	—	
Pooled fetal serum	Rh <sub>1</sub>	±	—	—	—	—	—	—	—	—	1	10
	Rh <sub>2</sub>	±	±	±	+	+	±	—	—	—	20	
	rh	—	—	—	—	—	—	—	—	—	—	

Comparison of the conglutinin activity of plasma at various ages showed significant differences, particularly during the fetal and neonatal periods (*cf.* Table III) as compared with adult plasma. It is interesting that the findings obtained in these experiments confirm the prediction made by one of us (3) purely on theoretical grounds. Since blocking antibodies (or glutinins) behave as if they are univalent and agglutinins behave as if they are bivalent, it was reasoned that the former are probably comprised of smaller molecules. This idea has been confirmed by demonstrating (13, 14) that blocking antibodies traverse the placenta more readily than agglutinins. To account for the abrupt onset of icterus gravis shortly after birth, it was postulated that the plasma of the fetus *in utero* is deficient in conglutinin, but that conglutinin increases in concentration abruptly after birth and is adsorbed by the infant's red blood

cells which are already coated with univalent maternal antibody acquired from the mother while *in utero*. The observations shown in Tables III, IV, and V support this hypothesis.

Table III demonstrates the greater potency of plasma and serum from adults as compared with plasma and serum from fetuses, when used as a source of conglutinin. When cord plasma or serum is used, not only are the titers considerably lower but the clumping is much less pronounced and more easily

TABLE IV

*Conglutinin Content of the Serum and Plasma of an Erythroblastotic Infant before and after an Exchange Transfusion, Using Blood with Half the Plasma Replaced by Saline Solution*

Material tested	Test cells	Dilutions of anti-Rh serum (patient J.)								Titer	Average titer
		Undiluted	1:2	1:4	1:8	1:16	1:32	1:64	1:128		
Serum of baby D., at beginning of transfusion	Rh <sub>1</sub>	—	—	—	—	—	—	—	—	0	2
	Rh <sub>2</sub>	++	++	+	±	—	—	—	—	4	
	rh	—	—	—	—	—	—	—	—	—	
Serum of baby D., at end of transfusion	Rh <sub>1</sub>	±	—	—	—	—	—	—	—	½	2½
	Rh <sub>2</sub>	±±	±±	+	±	—	—	—	—	1	
	rh	—	—	—	—	—	—	—	—	—	
Plasma of baby D., 6 days after transfusion	Rh <sub>1</sub>	++	++	++	++	±±	—	—	—	20	22
	Rh <sub>2</sub>	++±	++±	++±	++±	++	—	—	—	24	
	rh	—	—	—	—	—	—	—	—	—	
Pooled adult plasma	Rh <sub>1</sub>	++±	++	++	++	++	±±	—	—	40	60
	Rh <sub>2</sub>	++++	++++	++±	++	++	±±	±±	—	80	
	rh	—	—	—	—	—	—	—	—	—	
Pooled adult serum	Rh <sub>1</sub>	++±	++	++	++	+	—	—	—	16	20
	Rh <sub>2</sub>	++	++	++	++	±±	±	—	—	24	
	rh	—	—	—	—	—	—	—	—	—	

broken up by shaking. As has already been mentioned, conglutinin seems to consist of a colloidal complex of plasma proteins probably identical with the so called X-protein. Accordingly, the conglutinin content of serum or plasma would be influenced by the concentration of the plasma proteins. Thus, the weaker conglutinating activity of cord plasma and serum corresponds with the lower concentration of plasma proteins in the fetal blood (15).

Direct evidence supporting our concept of the pathogenesis of erythroblastosis was obtained by studying the conglutinin content of plasma from erythroblastotic infants who were treated by exchange transfusion (17, 18). When whole citrated blood was transfused, it was found that the total protein concentra-

tion in the infant's serum rose from a value of approximately 5.5 gm. per 100 cc. at the beginning of the transfusion to about 7 gm. per 100 cc. at the end of the transfusion,<sup>1</sup> and there was a simultaneous rise in the congenitinin content of the serum. On the other hand, when blood was transfused from which about two-fifths of the plasma had been removed and replaced with saline, there was no appreciable change in the total protein or congenitinin content. For example, in Table IV, are shown results of such studies on an infant with severe erythroblastosis (icterus gravis) treated by exchange transfusion at the age of 24 hours. In this case, blood from which part of the plasma had been removed and

TABLE V

*Conglutinin Content of the Serum of an Erythroblastic Infant before and after an Exchange Transfusion, Using Whole Citrated Blood*

Material tested	Test cells	Dilutions of anti-Rh serum							Titer	Average titer
		Undiluted	1:2	1:4	1:8	1:16	1:32	1:64		
Serum of baby L., at beginning of transfusion	Rh <sub>1</sub>	—	—	—	—	—	—	—	0	0
	Rh <sub>2</sub>	—	—	—	—	—	—	—	0	
	rh	—	—	—	—	—	—	—	—	
Serum of baby L., at end of transfusion	Rh <sub>1</sub>	±±	—	—	—	—	—	—	1½	1½
	Rh <sub>2</sub>	±±	±	—	—	—	—	—	1½	
	rh	—	—	—	—	—	—	—	—	
Serum of baby L., 2 days after transfusion	Rh <sub>1</sub>	++	++	++	+	—	—	—	8	8
	rh	—	—	—	—	—	—	—	—	
Pooled adult plasma	Rh <sub>1</sub>	++	++±	++±	++±	±±	±	—	20	18
	Rh <sub>2</sub>	++±	++±	++	++	+	—	—	16	
	rh	—	—	—	—	—	—	—	—	

replaced with saline was used for treating the infant, and it will be seen that there was no appreciable change in the congenitinin titer after the transfusion. This infant was very ill when first seen—icterus index 60 units, hemoglobin concentration 8.7 gm. per 100 cc., nystagmus and subconjunctival hemorrhages, spleen firm with edge three fingers below the costal margin, skin mottled due to hemorrhage and jaundice, mother Rh-negative with an anti-Rh titer of 20 units by the agglutination method and 70 units by albumin-plasma congenitination method (16)—but the infant made a complete recovery after the exchange transfusion. As shown in Table IV, tests done 6 days after the transfusion revealed a marked increase in the congenitinating activity of the infant's plasma,

<sup>1</sup> These chemical determination were made by Dr. Paul Riedel, Senior Chemist, Bellevue Hospital, New York City.

and there seems to be no doubt that the disease would have progressed to a fatal termination under the older method of treatment. This case may be contrasted with another treated early in our series before it was our practice to remove part of the plasma from the donor's blood and replace this with saline solution (16, 17). As shown in Table V, in the earlier case transfused with whole citrated blood, there was a significant increase in the conglutinating activity after the transfusion, and the disease progressed until the infant died on the 6th day, despite the fact that treatment had been instituted without delay after delivery of the infant by Caesarian section and while the clinical manifestations were still mild. On the basis of this case we introduced the practice of removing part of the donor's plasma and replacing it with saline solution. While the evidence provided by Tables IV and V may seem episodic, the satisfactory results we have obtained in a series of fifteen exchange transfusions carried out with blood with part of its plasma replaced by saline solution appear to justify this practice.

*Fortification of Conglutinin in Plasma by the Addition of  
Albumin and Globulin*

In order to throw further light on the nature of conglutinin, experiments were carried out to determine the effect on the conglutinating activity of plasma of the addition to it of albumin and globulin.

It was first found that the addition of certain optimal quantities of albumin markedly enhanced the activity of the plasma and this in fact led us to the development of a new direct test for Rh sensitization (16). To demonstrate the fortifying effect of added albumin on the conglutinating activity of plasma a series of mixtures of 25 per cent human albumin solution and pooled plasma were prepared in the following proportions: 9 parts of 25 per cent albumin solution and 1 part of pooled plasma, 3 parts albumin solution and 1 part of pooled plasma, equal parts albumin solution and plasma, 1 part albumin solution and 3 parts plasma, etc. (cf. Table VI). The conglutinating activity of each of these mixtures was determined and compared with that of the 25 per cent albumin solution and the pooled plasma. It will be seen that the lowest titer was obtained with the concentrated albumin solution (25 units), while the titer given by the pooled plasma was more than twice as high (64 units).<sup>2</sup> Yet by mixing these two ingredients in the proper proportions, a preparation could be obtained which yielded titers as high as 240 units. The titer values form a smooth curve, the optimal results being obtained with a mixture of 1 part of

<sup>2</sup> This finding supports our view that pooled plasma is a much more satisfactory medium than concentrated albumin solution for the conglutination test. Moreover, the albumin solution is more costly than plasma, less available, and very viscous, and therefore more difficult to work with.

the albumin solution and 3 parts of plasma.<sup>3</sup> The fact that a certain quantity of albumin gives optimal results in fortifying plasma in the conglutination test falls in with the concept that the added albumin becomes part of the complex which makes up conglutinin or X-protein, and does not accord with Diamond and Denton's (19) suggestion that the activity is determined merely by the protein concentration.

Similar results were obtained in experiments on the fortifying effect of added gamma globulin on the conglutinating activity of plasma. Since the gamma globulin fraction contains antibodies, including the isoagglutinins alpha and beta, the ideal preparation to use would be one made from the plasma of an

TABLE VI

*Comparison of the Conglutinating Activities of Different Mixtures of a 25 Per Cent Albumin Solution and Pooled Plasma*

Mixtures of human albumin and plasma (No. of parts)		Dilutions of anti-Rh serum (patient R.)										Control test with type rh cells	Titer
Albumin (25 per cent solution)	Plasma (pooled)	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512			
1	0	++	++	++	++	±	—				—	24	
9	1	+++±	+++±	+++±	+++±	±±	—				—	40	
3	1	+++±	+++±	+++±	+++±	±±	±±	—			—	80	
1	1	+++±	+++±	+++±	++	++	++	±±	±	—	—	196	
1	3	++	+++±	+++±	++	++	++	±±	+	—	—	256	
1	9	+++	+++±	+++±	++	++	±±	+	—		—	128	
1	27	+++	+++	+++±	++	++	±±	+	—		—	128	
1	81	+++±	+++±	++	++	±±	±±	±	—		—	96	
0	1	+++±	+++±	++	++	++	+	±	—		—	64	

Rh-positive group AB individual. Unfortunately such a preparation was not available to us and our experiments had to be carried out with purchased immune globulin,<sup>4</sup> which had a protein concentration of about 9.2 gm. per 100 cc.<sup>5</sup> A series of mixtures of this globulin solution with pooled plasma were prepared, just as in the experiment with albumin solution, and each mixture was tested for its conglutinating activity. The globulin solution itself gave very unsatisfactory results in the conglutination test; it was even more viscous and difficult to work with than the albumin solution and gave a titer of only 4 units in comparison to the titer of 48 units given by the pooled plasma. Yet as shown in Table VII, the addition of small quantities of the globulin solution

<sup>3</sup> Identical results were obtained when 30 per cent bovine albumin solution was substituted for the 25 per cent human albumin solution in the experiments.

<sup>4</sup> Cutter Laboratories.

<sup>5</sup> As determined by Dr. Paul Riedel, Senior Chemist, Bellevue Hospital, New York City.

to plasma served to increase the conglutinating activity of the latter. As in the experiments with albumin solution, the titers obtained yielded a fairly smooth curve, the maximal titer (128 units) being obtained when 1 part of globulin solution was mixed with 3 parts of plasma. The fact that a specific amount of globulin gives the optimal result, while the addition of a greater, or a smaller quantity gives a lower conglutination titer, indicates that the added globulin becomes a part of the X-protein complex, and again proves that the conglutinating activity does not depend merely on total protein concentration.

These results suggested that a further increase in the sensitivity of the conglutination test might be achieved with the aid of the globulin solution. As has already been indicated, a mixture of 4 parts plasma with 1 part of 25

TABLE VII

*Comparison of the Conglutinating Activity of Different Mixtures of a 9.2 Per Cent Gamma Globulin Solution and Pooled Plasma*

Mixtures of gamma globulin solution and plasma (No. of parts)		Dilutions of anti-Rh serum (patient R)								Control test with type rh blood	Titer
Globulin solution	Pooled plasma	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256		
1	0	±	+	±	—	—	—	—	—	—	4
9	1	+++	++	+±	±	—	—	—	—	—	12
3	1	+++	++	++	+±	+	—	—	—	—	32
1	1	+++	++	++	++	+±	+	±	—	—	64
1	3	+++	++	++	++	+±	+±	+	—	—	128
1	9	+++	+++±	++	++	+±	+±	—	—	—	80
1	27	+++	+++	++	++	+±	±	—	—	—	48
1	81	++	++	++	+±	+±	±	—	—	—	48
0	1	+++±	+++±	++	++	+±	±	—	—	—	48

per cent albumin solution yields titers about 3 to 4 times as high as those obtained with unmodified pooled plasma. If to such a mixture about one-fourth its volume of the concentrated immune gamma globulin solution is added, a still further increase in activity can be achieved, as evidenced by a twofold increase in the titers obtained in the conglutination test. Unfortunately, however, the globulin preparation which was available has the property of giving some weak non-specific reactions, even with group O cells, presumably owing to the presence of some irregular isoantibodies. These experiments have therefore been discontinued temporarily until a better preparation becomes available, namely one from Rh-positive group AB individuals.

*Studies on the Conglutinating Activity of Mixtures of Solutions of Albumin and Gamma Globulin*

The purpose of the next experiments was to determine whether it is possible to produce conglutinin synthetically by mixing suitable quantities of solutions



of purified albumin and gamma globulin. We had available the 25 per cent solution of human albumin and immune gamma globulin solution used in the tests just described.

In order to determine the minimum concentration of pure albumin in solution that exhibited any agglutinating activity the 25 per cent solution was mixed with saline in the following proportions: 9 parts albumin solution and 1 part saline solution, 8 parts albumin and 2 parts saline, 7 parts albumin and 3 parts saline, etc., and the agglutinating activities of these mixtures were compared with one another and with the original albumin solution, as well as with pooled plasma and serum (*cf.* Table VIII).

TABLE VIII

*Effect of Dilution of a 25 Per cent Solution of Human Albumin on Its Agglutinating Activity*

Proportion of albumin solution and saline		Dilutions of anti-Rh serum (patient R.)							Control test with type rh cells	Titer
Albumin (25 per cent solution)	Saline	1:2	1:4	1:8	1:16	1:32	1:64	1:128		
10	0	++	±	±	±	—			—	12
9	1	+++	+++	++	±	—			—	20
8	2	±	±	++	++	—			—	24
7	3	±	++	++	++	—			—	24
6	4	±	±	±	±	—			—	20
5	5	±	+	±	—				—	4
4	6	—	—						—	0
Pooled adult serum control.....		+++	++	++	++	—			—	24
Pooled adult plasma control.....		+++	+++	+++	+++	±	±	—	—	80

It appears that albumin solutions with concentrations less than 12.5 per cent have little or no agglutinating activity. Above 12.5 per cent concentration, the agglutinating activity increases and reaches a maximum at a concentration of about 17.5 per cent, while at still higher concentrations the agglutinating activity again diminishes. It is possible that the low titers obtained with the more concentrated albumin solutions were due to the great viscosity of such solutions. In any event the maximum titer obtained with any solution of albumin barely reached the titer obtained with pooled plasma. Since pooled plasma on the average has a total protein concentration of about 7 gm. per 100 cc., this again demonstrates that the agglutinating activity does not depend merely upon the total protein content.

Similar results were obtained when the immune globulin solution was diluted with saline solution. As shown in Table IX, on the basis of protein concentration gamma globulin has a much higher agglutinating activity than albumin, because a 2.8 per cent gamma globulin solution (mixture of 3 parts of the glob-

ulin solution with 7 parts of saline) was still weakly active (titer 4 units), while as already mentioned, solutions of albumin of less than 12.5 per cent strength are practically inactive. As in the case of the albumin solution, the activity of the globulin solution does not depend merely upon the protein concentration, because there is an optimal concentration above which the conglutinin titer decreases. We obtained the maximum titers with a globulin solution of approximately 7 per cent strength. It seems likely that the decrease in titer with higher concentrations of globulin is due, at least in part, to the marked viscosity of such concentrated solutions. The highest titer (48 units)

TABLE IX

*Effect of Dilution of an Immune Gamma Globulin Solution on Its Conglutinating Activity*

Proportion of globulin solution and saline		Dilutions of anti-Rh serum (patient J.)								Control test with type rh cells	Titer
Globulin (9.2 per cent solution)	Saline	Undiluted	1:2	1:4	1:8	1:16	1:32	1:64	1:128		
10	0	+++±	+++±	++++	++	++	—	—	—	—	24
9	1	++++	++++	++++	++	++	+±	—	—	—	40
8	2	++++	+++±	+++±	++	++	+±	±	—	—	48
7	3	+++±	+++±	++	++	++	+±	—	—	—	40
6	4	+±	+±	+±	+±	+±	±	—	—	—	24
5	5	+±	+±	+±	±	—	—	—	—	—	6
4	6	+	+±	+±	±	—	—	—	—	—	6
3	7	±	+	±	±	—	—	—	—	—	4
2	8	—	±	—	—	—	—	—	—	—	1
Pooled adult serum control. . . . .		++	++	++	++	+±	±	—	—	—	24
Pooled adult plasma control. . . .		++++	+++±	+++±	++	++	+±	+	—	—	64

given by any globulin solution was higher than that of the pooled serum control (24 units) but less than that of the pooled plasma control (64 units).

It will be noted that when the 25 per cent albumin solution was diluted with an equal volume of saline solution it was almost devoid of conglutinating activity and the globulin solution was only weakly active when similarly diluted. Starting with these two dilute solutions, namely, 12.5 per cent albumin solution and 4.6 per cent globulin solution, a series of mixtures were prepared in the following proportions: 27 parts globulin solution and 1 part albumin solution, 9 G and 1 A, 3 G and 1 A, 1 G and 1 A, 1 G and 3 A, 1 G and 9 A, and 1 G and 27 A, and the conglutinating activity of each of these mixtures was then determined and compared with that of pooled human plasma. As shown in Table X, despite the relative inactivity of the original solutions, mixtures were

obtained with high conglomerating activities, even exceeding that of pooled plasma. The titers obtained yielded a relatively smooth curve and the maximum titers were obtained with the mixtures 3 G and 1 A, and 1 G and 1 A. The protein content of each mixture was then calculated in terms of albumin, gamma globulin, and total protein. It will be seen that the mixtures which gave the highest conglutination titers had total protein concentrations of 6.6 to 8.6 gm. per 100 cc., which is equal to the protein concentration of normal serum. If, based on the above findings, we assume that the optimal results would be given by a mixture of 2 G and 1 A we find that this would correspond to a total

TABLE X

*Conglutinating Activity of Mixtures of Solutions of Purified Albumin and Gamma Globulin*

Mixtures of 4.6 per cent globulin solution and 12.5 per cent albumin solution (No. of parts)		Dilutions of anti-Rh serum (patient R.)										Control test with type rh cells	Titer	Protein content		
Globulin solution	Albumin solution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024			Alb.	Glob.	Total
														gm./100 cc.	gm./100 cc.	gm./100 cc.
1	0	+	++	+++	+	-						-	16	0	4.6	4.6
27	1	++	++	++	++	++	+					-	64	0.5	4.4	4.9
9	1	++	++	++	++	++	++	++	++			-	320	1.3	4.1	5.4
3	1	++	++	++	++	++	++	++	++	++		-	394	3.1	3.5	6.6
1	1	++	+++	++	++	++	++	++	++	++	+	-	394	6.3	2.3	8.6
1	3	+++	+++	+++	+++	+++	++	++	-			-	96	9.4	1.1	10.5
1	9	++	++	++	++	++	+					-	24	11.3	0.5	11.8
1	27	±	±	++	±	±	-					-	12	12.0	0.2	12.2
0	1	±	-									-	1	12.5	0	12.5
Pooled human plasma.....		++	++	++	++	++	++	±	-			-	96	4.6*	2.3*	6.9*

\* Hypothetical

protein concentration of 7.2 gm. per 100 cc. partitioned into 4.2 gm. albumin and 3.0 gm. globulin per 100 cc. This is remarkably close to the albumin-globulin ratio of normal human serum.

Certainly these results support the concept of the identity of X-protein and conglutinin. The fact that 3 G and 1 A, and 1 G and 1 A mixtures gave titers approximately 4 times as high as our normal plasma control rather exceeds expectations, considering that no fibrinogen was used in preparing these mixtures. This would indicate that normal plasma contains other substances which tend to maintain the albumin and globulin molecules in solution and to prevent their combination or aggregation to form X-protein. Another possibility is that in the preparation of the purified albumin and gamma globulin solutions these proteins were somewhat denatured, with diminution of their hydrophilic qualities and increase in their tendency to form aggregates.

## COMMENT

The work of Pedersen and the results presented in this paper show that conglutinin and X-protein are substances which can be titrated and weighed. The main test for a new hypothesis is the ability to predict from it correctly hitherto unknown facts, which can be tested by direct experimentation, and to open new fields of research. As shown in the present paper, the theory of conglutination has led to new, more sensitive tests (16) for univalent antibodies and has resulted in the prediction, now substantiated, that conglutinin can be formed *in vitro* by mixing solutions of albumin and gamma globulin in proper proportions. It has also led to a new theory of pathogenesis of erythroblastosis fetalis which is already supported by a considerable body of experimental data (20, 21). Moreover, these new concepts have increased the accuracy of the prenatal prediction not only of the occurrence of erythroblastosis fetalis, but also of the type and severity of the disease, and has led further to a more rational and successful treatment of the disease by exchange transfusion (17, 18).

While the albumin test of Diamond and Denton (19) and the anti-globulin test of Coombs *et al.* (22) are ingenious, they are less sensitive and entail the use of special materials not available to the average laboratory. These tests have the disadvantage that they entail artificial conditions having no counterpart *in vivo*, and therefore yield little or no insight into the nature of their actions of univalent antibodies.

## SUMMARY

1. Dilution of pooled plasma with more than an equal volume of saline solution destroys its ability to produce conglutination of red cells sensitized by univalent antibody. This can be correlated with Pedersen's work showing that X-protein is readily dissociated by dilution. The observation explains the discrepancy between the reports of British and American workers regarding the incidence of Rh "agglutinins" in the serum of Rh-negative mothers of erythroblastotic babies.

2. Plasma has a higher conglutinating activity than serum as shown by the finding that plasma gives titers on the average more than twice as high as those obtained with serum. The greater activity of plasma would seem due to the presence of fibrinogen which is apparently an important component of the colloidal complex of plasma proteins making up conglutinin.

3. Aside from its action in precipitating fibrinogen, heating at 56°C. for one-half hour has no harmful effect on conglutinin.

4. Fetal plasma and serum yield much lower conglutination titers than adult plasma and serum, indicating that fetal blood is deficient in conglutinin. After birth, there is generally a marked increase in the conglutinin content of the blood. There is little or no variation in the conglutinin activity of sera from different normal adult individuals.

5. The use of whole citrated blood in exchange transfusion to an erythroblas-

totic baby caused an appreciable rise in the total plasma proteins after the transfusion and a corresponding increase in the conglutinating activity. When however, in another instance, two-fifths of the plasma was removed from the donor's blood and replaced with saline, there was no appreciable change in the protein concentration or conglutinin activity of the infant's plasma after the transfusion.

6. The fortification of pooled plasma by mixing 4 parts of it with 1 part of 25 per cent human albumin solution markedly increased its conglutinin content as shown by a fourfold increase in the conglutination titers obtained. Addition of less or more than this optimal amount of albumin resulted in lower titers. The 25 per cent human albumin solution itself yielded titers only half as high as did unmodified pooled plasma and was difficult to work with because of its high viscosity. Similar results were obtained in experiments with immune globulin solutions and pooled plasma.

7. Albumin solutions of less than 12.5 per cent concentration had little or no conglutinin activity; similarly, immune globulin solutions of less than 4.6 per cent concentration gave only relatively low titers when used as conglutinin. Yet, mixtures of these dilute solutions in certain optimal proportions yielded solutions with conglutinin activities considerably higher than that of pooled plasma. The albumin-globulin ratio in the mixtures giving the best results proved to be approximately the same as the albumin-globulin ratio of normal human serum or plasma.

8. Suitable mixtures of albumin and globulin solutions with a total protein concentration equal to that of normal plasma gave conglutination titers about four times as high as those obtained with unmodified pooled plasma. This suggests that there may be substances in normal plasma which tend to maintain the albumin and globulin in molecular dispersion. Another possibility is that in the fractionation process the albumin and globulin are rendered less hydrophilic, thus increasing their tendency to form colloidal aggregates.

9. The experiments described support the theory that clumping of cells by univalent antibodies in plasma media occurs in two stages, namely, (1) specific adsorption of univalent antibodies, and (2) non-specific adsorption of conglutinin by the sensitized cells causing them to stick together. The experiments further support the concept of conglutinin or X-protein as a colloidal aggregate of plasma proteins. Finally, they demonstrate that the intensity of the clumping (conglutination—not agglutination) depends on the quantity and quality of conglutinin and not merely on the total protein content of the medium of suspension.

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# THE FINE STRUCTURE OF CLOTS FORMED FROM PURIFIED BOVINE FIBRINOGEN AND THROMBIN: A STUDY WITH THE ELECTRON MICROSCOPE\*

BY CLINTON VAN ZANDT HAWN, M.D.,† AND KEITH R. PORTER, Ph.D.

(From the Department of Pathology, Harvard Medical School, Boston, and the Laboratories of The Rockefeller Institute for Medical Research, New York)

PLATES 29 TO 31

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The electron microscope can provide information on the fine structure of blood clots which may be useful in the consideration of the over-all physical-chemical description of the clotting mechanism. Wolpers and Ruska (1) (1939) presented the results of their studies of fibrin produced from whole blood plasma. Subsequently (2) they studied the pellicles from the spinal fluids of cases of tuberculous meningitis and noted the same general structure as had been encountered in the clots of blood plasma. These they described as being made up of micellar bundles, often arranged in parallel to form thicker strands which were held in a network by the intercommunicating branches. They also described cross-striations in the micellar bundles and in the composite fibers; these striations showed periodicities ranging from 200 to 350 Å. No similar striations were found in clots from blood plasma and it was concluded that striations were a characteristic of the fibrin of the spinal fluid pellicles. Schmitt and Hobson (3) investigated similar spinal fluid pellicles and found striations in preparations from one case of seven investigated.

The methods for isolating and purifying proteins of the blood plasma, both human and bovine, developed in the Department of Physical Chemistry of Harvard Medical School by Cohn and his associates (4-7) have made available certain of the proteins in states of greater purity and in greater quantity than had been obtained by previous methods. Along with others of these, fibrinogen and thrombin have been quite extensively studied and it is known that the mechanical properties of the clots formed from them can be varied within wide limits by control of such factors as pH, the temperature at which the interaction of fibrinogen and thrombin takes place, the concentrations of the reacting substances and the presence of various chemical substances (8). In the course of investigations on the culture and behavior of tissue cells in clots from purified bovine fibrinogen and thrombin (9), it seemed desirable to make some parallel studies on the influence of the above factors on the fine structure of the clot.

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† Junior Fellow, the Society of Fellows, Harvard University, Cambridge.

The observations in this initial report are primarily concerned with the effects of alterations of pH.

### *Materials and Methods*

The fibrinogen and thrombin preparations used in these experiments were generously provided by the Chemical Research and Development Department of Armour and Company, Chicago. The fibrinogen (lot C-185A) contained 79 per cent clottable nitrogen and was furnished in the form of a dried powder of which approximately 40 per cent by weight was sodium citrate. The thrombin (lot P-38) had a clotting activity of 27 units<sup>1</sup> per mg. of total weight. These substances were dissolved in buffered physiological saline or in Tyrode's solution as indicated in the figure legends. Determinations of the pH were made with the glass electrode. The solutions were allowed to react at room temperature (22.1–23.0°C.).

For convenient study by means of the electron microscope it was desirable to obtain a clot sufficiently thin to permit a clear resolution of individual fibers and yet thick enough to provide a picture of the tridimensional orientation of the fibrin strands. The following technique was devised. Its results have been entirely reproducible, and satisfactory for the observations thus far undertaken:—

Conventional glass microscopic slides (1 inch × 3 inch) were coated with a thin film of formvar<sup>2</sup> by immersing them in a 0.15 per cent solution of this material in ethylene dichloride, and allowing the solvent to evaporate. The resulting film was approximately 200 Å in thickness.

A clotting mixture was prepared by the addition of the thrombin in buffered saline solution at a concentration of 10 units per cc. to the fibrinogen in similarly buffered solution at a concentration of 0.06 per cent. To 5 cc. of the fibrinogen solution in a short test tube, 0.5 cc. of the solution of thrombin was added and mixed rapidly by pipetting. The coated slide was immersed in the clotting mixture for 15 seconds, then removed and placed horizontally over water in a covered Petri dish. The clotting process was allowed to proceed undisturbed for 2½ minutes. At the end of this time the clot in the test tube was well formed, as was the film of clot on the coated slide. The latter was then flooded with a solution of phosphotungstic acid (11), 0.2 per cent, for approximately 15 minutes. At the end of this time, the phosphotungstic acid was removed by flooding the slide with distilled water. While under water, small portions of the clot-covered film of formvar were peeled away from the glass and mounted on the conventional 3 mm. discs of wire cloth used to support objects for electron microscopy. The preparations were then quickly drained and dried and examined.

The clot from the test tube was removed and compressed to obtain free fluid for pH determinations. It was assumed that the measurement of the pH of the clot in the test tube, which was the source of the clot on the microscope slide, gave at least a first approximation to the pH value of the latter. The measurements of pH of the various solutions were recorded throughout, but those of the fluid obtained from the final clots were considered to be the most significant.

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<sup>1</sup> The unit is defined as the amount required to clot 1 cc. of a standard fibrinogen solution in 15 seconds (8) or the amount which clots a 1 per cent solution of fibrinogen in a test tube 1 cm. in diameter at 25°C., pH 6.3, in approximately 45 seconds (10).

<sup>2</sup> This is a polyvinyl formal resin, sold by the Shawinigan Products Corporation. It seems to be particularly valuable for making preparations by these methods, presumably because of its tensile strength. It is insoluble in H<sub>2</sub>O and probably does not interact with the fibrinogen or thrombin or influence the character of the formed clot.



The buffers used were freshly prepared Sørensen's phosphate mixtures and in two experiments the fibrinogen was dissolved in Tyrode's solution (Earle's modification). The alteration to acidity was obtained in the latter by maintaining a partial tension of  $\text{CO}_2$  over the solutions and clotting mixture. In some experiments the vapors of osmium tetroxide were used in place of the phosphotungstic acid, with no marked variation in the results.

An RCA electron microscope, type E.M.U., was used for all microscopy.<sup>3</sup> Most of the micrographs were taken at magnifications between 5000 and 7000 and enlarged photographically. Particular care was taken to use an electron beam of minimal intensity so as to avoid, as far as possible, any alterations in the material.

### Observations

In an attempt at an orderly presentation of data we have arbitrarily subdivided the description of certain of the characteristics of the clots formed at three different values of pH. The measurements represent mean values derived from the study of many micrographic fields in the several preparations made at each of the several pH intervals studied. A more nearly statistical analysis is in preparation, but the approximations here recorded seem adequate for the purpose of an initial presentation.

*Coarse Structure of the Fibrin Network.*—The general architecture of the clots is illustrated in Figs. 1 to 4. It appears that under the conditions of these several experiments the unit of structure is an elongated fiber (to be called a unit fiber), and that such fibers are joined laterally in various ways to form compound fibers (Fig. 5). This results in a tridimensional network of branching strands. Studies of the micrographs also show evidence of the tapering character of the unit fibers. Many of these appear to have their tapered tips incorporated in incompletely polymerized material on the plastic film.

In the clots formed at pH 8.5 (Fig. 1), most of the fibers are single and show relatively infrequent fusion with other single or unit fibers so that but few compound fibers are formed (Fig. 1). The fibers are twisted and curved. The interstices are, on the average, very small.

At pH 7.6 the formation of more numerous compound fibers is apparent (Fig. 2). These are much less curved, particularly over the intervals where two or more unit fibers lie bundled in parallel longitudinal association. The interstices are generally larger.

The micrographs of clots formed at pH 6.3 show the more generalized formation of compound fibers, and only a few independent unit fibers (Figs. 3 and 4). The broad compound fibers are often straight and the interstices vary widely in size and shape but are of larger average size. The number of fibers in the broad strands varies from place to place as component fibers branch from the main bundles to join other single or compound fibers. As many as eight unit strands can be demonstrated in some of the broad compound fibers. Often the point

<sup>3</sup> The authors are grateful to Dr. R. M. Taylor for permission to use the instrument belonging to the laboratories of the International Health Division of The Rockefeller Foundation.

of juxtaposition of several fibers appears as a *nidus* from which multiple branchings appear to be derived (Fig. 6).

The average values of the diameters of representative unit fibers formed at these different pH values are indicated in Table I. These were derived from measurement of separate, single fibers and also units within compound fibers where the numbers and limits of the components could be made out with certainty.

It is evident that the greater the hydrogen ion concentration in the clotting mixture the larger the average diameter of the unit fiber, single or in bundles. The larger unit fibers at the lower pH, and particularly the frequent close lateral association of two or more other unit fibers produce the characteristic coarse architectural features described.

It is also of interest to note that in the background of the micrographs there was a significantly greater amount of amorphous unpolymerized protein precipitate in the preparations clotted at pH 8.5 than in those clotted at pH 6.3.

TABLE I

pH.....	6.3	7.6	8.5
m $\mu$ .....	60	49	36

*Fine Structure of the Individual Fibers.*—Examination of the individual fibers in the electron micrographs (Figs. 4 to 7) shows a striking cross-striated appearance essentially similar to that noted only in human spinal fluid pellicle fibrin by Ruska and Wolpers (2). This is due to alternate bands of relatively high and low density to electrons. These striae are more readily seen in the fibers formed at the acid pH than in those formed at the alkaline pH. While most of our preparations were treated with phosphotungstic acid, it is to be noted that these periodic striae were clearly visible as well in preparations fixed in vapors of osmium tetroxide. Also, in preparations covered with gold by the so called shadow-cast technique (12), some fibers show marginal prominences which correspond to the cross-striations (Fig. 7).

One of the most striking features of the fine structure is the coinciding striations of laterally associated unit fibers. Often this coincidence can be seen to extend across the several fibers of a large bundle. The longitudinal outlines of the individual fibers in such bundles are not lost (Figs. 5 and 6).

The tapered ends of the unit fibers show the characteristic striations, and signs of the same periodicity are to be seen in the incompletely polymerized material, particularly in the preparations clotted at pH 6.3. The fine fibrillar material visible in the background of the gold-shadowed preparation shows a comparable periodic beading (Fig. 7). Average lengths of the periods at three

different pH values are presented in Table II. In a few fields some of the fibers looked as if stretched. Measurements of the lengths of the periods of the cross-striations in these stretched fibers show them to be increased significantly.

There are certain technical difficulties encountered in making and analyzing the required measurements. Among these may be mentioned the lengthening of the distance from one dark band to the next as the apparent result of stretching. Also, there is a tendency for the periodicity to be more regular in compound fibers, and in these the distances between dark bands appear slightly shorter than in individual fibers. In addition, the striations are less well defined in fibers of clots formed at pH 8.5 than in those formed at pH 7.6 or pH 6.3. When these factors are taken into account, it seems improbable that the slight variations of the recorded average values of Table II have significance. In-

TABLE II

pH.....	6.3	7.6	8.5
Å.....	245	243	253

deed the average periodicity throughout the clots formed at the three values of pH is strikingly constant.

## DISCUSSION

Any attempt to describe the details of the mechanism of the clotting of whole blood from the observations recorded in this report would be premature and speculative. The method of study we have outlined has obvious limitations, particularly since the preparations examined in the electron microscope have been dehydrated in the vacuum of that instrument and certain distortions and contractions must be assumed to have taken place. Furthermore, the system studied has been simplified by the use of the two protein substances assumed to interact in the final stage of the clotting mechanism, and only the effect of alterations of pH has been considered in this initial report. Nevertheless, the general appearance of the clots is essentially similar to that of those studied by Wolpers and Ruska (1, 2) who used as objects clots formed from whole blood, plasma, and spinal fluid under more nearly physiological conditions.

Ferry and Morrison (10) studied the clotting of the solutions of human fibrinogen by the addition of human thrombin under various conditions of concentration of the reacting proteins, pH, ionic strength, temperature, and the addition of certain polyhydroxyl compounds, and they concluded that clotting is to be interpreted as a three-dimensional polymerization. They proposed as the structure of the fine clot a network of chains, consisting of

fibrinogen molecules joined end to end, cross-linked partly at least by primary chemical bonds. They suggested that the coarse clot is a network of bundles of such chains, cross-linked largely by secondary bonds and as result of lateral association. Our own observations tend to bear out in large part these hypotheses. However, at the concentrations of fibrinogen, thrombin, and hydrogen ion we have studied, and under the other described conditions of our experiments, it would seem that the fine, grossly transparent clot formed at pH 8.5 is composed of unit fibers of multiple, rather than single chains of fibrinogen molecules. The marked increase in the diameter of these unit fibers in clots formed at pH 6.3, and especially the tendency of the unit fibers to form compound fibers by lateral association could well account for the opacity of the gross clot at this pH. The tendency to form compound fibers may also explain in part the syneresis of coarse clots that is observable in the gross.

The striking regularity of the cross-striations of the fibers at the different values of pH studied is difficult of interpretation. Measurements of double refraction of flow, viscosity, osmotic pressure, and sedimentation in the ultracentrifuge of solutions of human fibrinogen have led to a tentative description of the model of the molecule as an elongated ellipsoid of revolution about  $35 \times 700 \text{ \AA}$ , with a molecular weight of about 500,000 (13).<sup>4</sup> Bovine fibrinogen recently has been subjected to osmometric and viscometric studies with essentially the same results (14). If it is assumed that the primary interaction of fibrinogen and thrombin results in an end-to-end linkage of such long molecules, with subsequent lateral association, it is not easy to reconcile the measured periodicity of the unit strands with the dimensions of the proposed molecular model. Possibly, in polymerization, the shape of the fibrinogen molecule is altered by extensive folding and consequent shortening. The region of greatest folding could be, as suggested for collagen (15), the region of greatest density; *i.e.*, the dark striation.

Study of the incompletely polymerized material to be seen in the backgrounds of the clots (Fig. 7) has yielded some evidence for the end-to-end linkage of elongated structures of the orders of magnitude of the proposed fibrinogen molecules. The limit of resolution in the better micrographs seems to lie between  $5 \text{ m}\mu$  and  $10 \text{ m}\mu$ . The finest chains visible show longitudinally arranged densities and irregularities and may represent single strands or "protofibrils" (15) of elongated molecules, linked end to end. Also, it would appear that the unit fibers are built up by the lateral association of several such chains (Fig. 7). In this association the regions of greatest density coincide in a remarkable way to give the characteristic banded or striated appearance. Even when the unit fibers come to lie in close association, some force operates to line up the striae so that they coincide directly across the compound strand (Figs. 5 and 6).

<sup>4</sup> Another model which could fit many of the data would be a disc-shaped ellipsoid of revolution  $10 \text{ \AA}$  in thickness with a diameter of  $350 \text{ \AA}$  (13).

A discussion of the nature of the forces involved in the precise lateral association of molecular chains and bundles of such chains (unit fibers) so that the striations correspond in fibers lying side by side is not within the scope of this report. However, as Ferry and Morrison (10) have pointed out, the conditions which favor decreasing coarseness of the fibers are those which would be expected to diminish attractive forces and the tendency to aggregation. Since the isoelectric point of human fibrinogen is at about pH 5.5, an increase of pH from 6.3 to 8.5 would increase the net charge of the molecule and hence the mutual electrostatic energy which might be expected to result in diminution of electrostatic attractive forces between the large molecules.

#### SUMMARY

1. A technique has been described for the preparation of clots from purified fibrinogen and thrombin of bovine origin which are suitable for study with the electron microscope. Experiments have been carried out to compare the fine structure of clots prepared at various values of pH.

2. The clots are composed of meshworks of single and compound fibers. At pH 8.5 the unit fibers have a smaller average diameter than those formed at pH 7.6 or pH 6.3. The tendency for the lateral association of unit fibers into compound fibers is markedly increased as the pH is decreased.

3. A striking feature of all the clots studied is cross-striation of the unit fibers. The periodicity of these striae is quite constant throughout (approximately 250 Å). There is a precise coincidence of the striations of the individual unit fibers where these are associated side by side to form compound fibers.

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## EXPLANATION OF PLATES

### PLATE 29

FIG. 1. Micrograph of clot formed at pH 8.5: in Tyrode's solution, bicarbonate buffer. Treated with 0.2 per cent phosphotungstic acid solution.

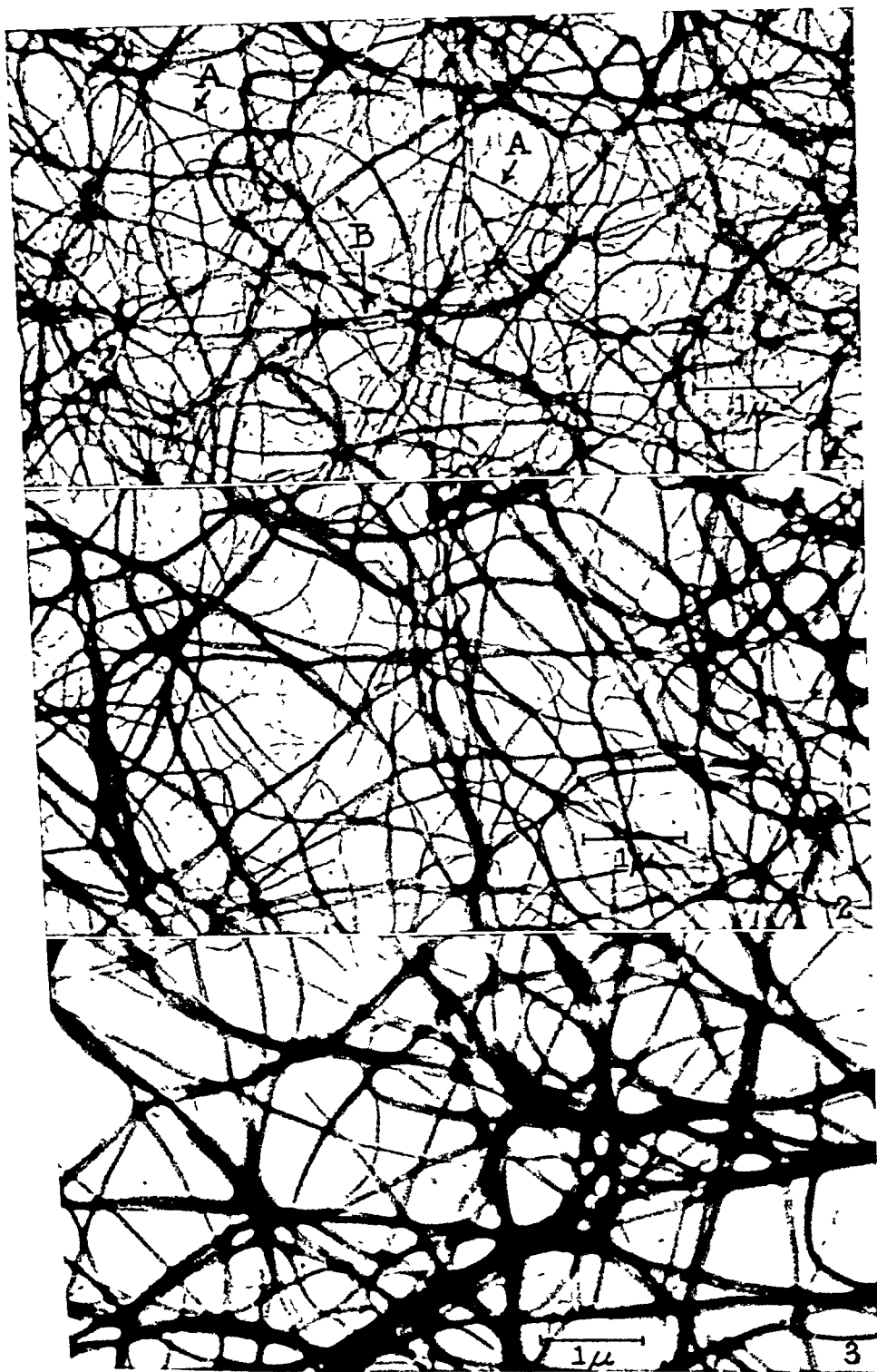
The unit fibers (A) are thin and often curved. Relatively few compound fibers (B) are formed by lateral association of the units. The interstices are fairly uniform and small. Much of the background material appears non-fibrous.  $\times 15,200$ .

FIG. 2. Micrograph of clot formed at pH 7.6: in 0.85 per cent saline, Sørensen's phosphate buffer. Treated with 0.2 per cent phosphotungstic acid solution.

The unit fibers are broader and many compound fibers are to be seen. These are less curved. The interstices vary widely in size and shape but many are larger than in Fig. 1. The background material is for the most part fibrillar.  $\times 15,200$ .

FIG. 3. Micrograph of clot formed at pH 6.3: in 0.85 per cent saline, Sørensen's phosphate buffer. Treated with 0.2 per cent phosphotungstic acid solution.

Many coarse strands formed of associated unit fibers characterize the clot. The unit fibers are broader than in Figs. 1. and 2. The interstices show marked variation in size and shape. Relatively little background material is visible and that which can be seen seems polymerized into long fibrils.  $\times 15,200$ .



### PLATE 30

FIG. 4. Micrograph of clot formed at pH 6.3: in 0.85 per cent NaCl, Sørensen's phosphate buffer. Treated with 0.4 per cent phosphotungstic acid solution.

This illustrates the branching of the compound fibers and the other general architectural features of the coarse clot. Characteristic cross-striations are visible in most of the compound fibers, as well as in the single fibers, including their tapering ends.  $\times 30,500$ .





(Hawn and Porter: Electron microscope study of clots)

### PLATE 31

FIG. 5. Micrograph of fibrin formed at pH 6.8: in Tyrode's solution, bicarbonate buffer. Treated with 0.2 per cent phosphotungstic acid solution.

The several compound fibers show the striking coincidence of the striations where the unit fibers lie in lateral association.  $\times 40,000$ .

FIG. 6. Micrograph of fibrin formed at pH 6.3: in 0.85 per cent NaCl, Sørensen's phosphate buffer. Treated with 0.4 per cent phosphotungstic acid solution.

Detail of striations showing striking coincidence across a flattened bundle formed by the lateral association of approximately eight unit fibers.  $\times 44,000$ .

FIG. 7. Micrograph of fibrin formed at pH 6.8: in Tyrode's solution, bicarbonate buffer. Treated with 0.2 per cent phosphotungstic acid solution. Gold-shadowed.

This preparation shows details of fiber structure. Fine fibrils lie in parallel with coinciding densities. Some of the resulting fibers appear flattened. It is evident also that the finely fibrillar background material shows a periodicity similar to that in the larger fibers.  $\times 50,000$ .



(Hawn and Porter: Electron microscope study of clots)



# THE RELATIVE PRESSURES WITHIN CUTANEOUS LYMPHATIC CAPILLARIES AND THE TISSUES

By PHILIP D. McMASTER, M.D.

*(From the Laboratories of The Rockefeller Institute for Medical Research)*

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The formation of lymph takes place in the main through the walls of the minute lymphatic capillaries, the larger channels acting chiefly, if not wholly, as conveyors of this fluid to the blood. To learn something about the forces which tend to promote the formation and flow of lymph in the smallest vessels, the lymph pressure existing within cutaneous lymphatic capillaries in the ears of mice has been compared in the present work with the pressure prevailing in the tissue immediately outside them. This has been done both under normal conditions and during the development of rapidly forming edema which greatly increases lymph formation, as is well known.

## *Techniques*

Most of the techniques employed have been those of earlier work in this laboratory (1-5). Improved methods were used for the measurement of edema fluid pressure (5) and for the estimation (4) of the approximate pressure in the normal cutaneous tissues, which do not contain enough free fluid for the interstitial fluid pressure to be estimated directly (6-8). Doubtless because of the fact that lymphatic capillaries, unlike blood capillaries, are not readily visible under the microscope, no direct measurements of the lymph pressure in lymphatic capillaries of the skin have been reported previously. However, in the course of other studies (10-15) from this laboratory, the difficulty had been overcome by the development of methods whereby the lymphatic capillaries are rendered visible as they pass through uninjured living skin. As a result, all that was lacking for the study of the pressure relationships inside the lymphatic capillaries and round about them was a suitable method for the measurement of fluid pressure in these channels.

## *The Determination of Pressure*

The work was carried out on visualized lymphatic capillaries in the skin of the ears of mice lightly anesthetized with nembutal,—0.5 mg. for each 10 gm. of body weight,—as in the previous studies (4). Under these conditions the circulation of the ears appears to be excellent, although the blood pressure is slightly reduced (9).

Fine glass tubes were inserted directly into the channels and the capillary pressures were measured manometrically. In the ear of the mouse lymphatic capillaries are larger as a rule than blood capillaries; channels suitable for intubation can be found varying between 25 and

100  $\mu$  in width. One can introduce into these channels glass tubes larger than the micro pipettes required for the measurement of pressure in blood capillaries, and for this reason they will be termed micro cannulae instead of micro pipettes. Despite their size, the measure of pressure in lymphatic capillaries by means of them is not simple for they must be large enough to fill the lumina of the channels so completely that no leakage of lymph occurs, and it is difficult to insert them without tearing the delicate walls of the capillaries, which are often flat and ribbon-like though relatively broad.

*Method and Apparatus.*—Soft glass micro cannulae were blown in various sizes, from 10 to 100  $\mu$  in external diameter. A single cannula, chosen as will be described, was firmly fixed horizontally in the holder of a Chambers micro manipulator and connected by flexible metal tubing to one end of a horizontally placed glass tube 6 mm. in diameter and about 10 cm. long, equipped with a stop-cock in its mid portion. The other end of the tube communicated by heavy rubber pressure tubing with a broad bottomed 300 cc. glass reservoir connected with two manometers. The bottle could be raised or lowered by a rack-and-pinion device. This part of the manometric apparatus has been fully described and illustrated in Text-fig. 1 and Figs. 1 and 2 of a previous paper (1). Suffice it to say that one of the manometers consisted of a glass tube about 2 mm. in interior diameter fixed at an angle of  $15^\circ$  with the horizontal, and filled with kerosene. Small differences in pressure could be measured with it. The second manometer was a simple vertical tube for measurement of pressures greater than that of a column of water 2 cm. high. This second manometer, the reservoir, the glass tube with the stop-cock, and the micro cannula were all filled with the fluid to be introduced into the lymphatic capillaries or into the tissues during the course of the test. Two identical apparatuses of this sort were used. The first was filled with an 0.2 per cent solution of vital red in Locke's solution, a mixture so diluted with water as to render it isotonic with blood. The other contained a mixture of 0.5 per cent of the blue dye, pontamine sky blue, in Locke's solution, to which there had been added 10 units of heparin to each 100 cc. This too was isotonic with blood, and as will be described below it served as a relatively unabsorbable fluid with which to make pressure measurements.

*The Visualization of Lymphatic Capillaries.*—The lymphatic capillaries had to be rendered visible before the pressure of the lymph could be determined. Two methods were used for this purpose. The first was suggested by some earlier unpublished studies in which large mice under nembutal anesthesia had been injected intravenously with fluid containing the highly indiffusible dye, vital red, in amounts too small to color the skin visibly to the naked eye. Many of the mice had small bites or other injuries at the periphery of their ears, and a local escape of dye took place from the blood vessels into the tissue about these wounds, giving rise to a narrow band of red at the ear margin. Animals in which this had occurred were placed on plastaline molds with the ears spread on porcelain plaques (16) so that the minute blood vessels and lymphatics could be viewed microscopically in the living animal. It was noted that after a few minutes some of the color passed from the tissues into the adjacent lymphatic capillaries rendering them visible, and thereafter drained to others with result that they too could now be seen. Lymphatics thus accidentally rendered visible were utilized for some of the present experiments while for others the margins of the ears of young animals were purposely injured by pinching them with forceps and the dye was injected into the blood several days later.

Unfortunately visualization of the lymphatics did not always take place under the circumstances outlined. For this reason another technique was frequently used: a modification of one already described in many previous papers (10–16). Mice were anesthetized with nembutal, their ears were spread on porcelain plaques, and under the binocular microscope the skin of the ear was punctured with an especially sharp micro cannula, attached to the apparatus that had been filled with the solution containing vital red. After the micro cannula had been pushed through the skin it was run parallel to the surface for a few millimeters in

order to tear a few lymphatic capillaries of the exceedingly rich plexus which lies in the sub-papillary layer of the corium (10, 11). Next, just enough pressure was put upon the red fluid in the apparatus to force about 1.0 c. mm. of it slowly into the tissues. Within a few minutes thereafter it generally entered one or several of the torn lymphatic capillaries and flowed 2 or 3 mm. further into other lymphatics separated from the torn ones by valves and receiving lymph from capillaries draining uninjured portions of the ear. One of these latter channels was selected for cannulation in the way now to be described:—

*The Pressure Measurement.*—The anesthetized mouse, with lymphatic capillaries visualized by one or the other method, was placed on a plastaline mold on a mechanical stage that could be moved in any direction and the visualized lymphatics were inspected under the microscope to find a segment containing one or more valveless tributaries. Having found one, the observer selected a micro cannula large enough to fit it so snugly that leakage of lymph would not occur after the insertion. This cannula was then attached to the manometric apparatus containing the mixture of blue dye, heparin, and diluted Locke's solution, filled with this fluid, and clamped in place so that its tip came into focus in the center of the microscope field. Its position was not altered thereafter. Next, with the manometers set at zero and the blue fluid in the apparatus and the cannula at atmospheric pressure, the stop-cock behind the cannula was closed. By appropriate movements of the mechanical stage and gentle traction on the margins of the ear, the stationary tip of the cannula was forced through the epidermis and into the chosen segment of the lymphatic capillary, in the direction of lymph flow. When leakage from the capillary occurred about it, and this was frequent, the escape of pink fluid could readily be seen under the microscope, and the preparation was discarded. If the procedure was successful the micro cannula filled the capillary and obstructed lymph flow. Then by further movement of the mechanical stage or gentle traction on the ear the tip of the cannula was thrust further, to a point just above, and distal, to the entrance of the valveless tributary into the cannulated vessel. The tributary normally supplied lymph to the portion of the cannulated channel below the cannula's tip, and lymph flow through it continued. Consequently, the pressures measured are to be regarded as lateral pressures.

As soon as the cannula was in place the stop-cock of the manometric apparatus was opened, establishing fluid continuity at zero pressure. Almost invariably the blue contents of the cannula were forced backwards towards the manometer by the pale pink fluid in the lymphatics. Pressure was then put upon the contents of the cannula by slowly raising the reservoir of the manometric apparatus by means of its rack-and-pinion device (1) until the blue fluid moved out into the lymphatic capillary. As soon as this had happened the pressure was quickly lowered a little, and by trial and error a point was found at which the boundary of the pink and blue fluids lay at the cannula's tip. The pressure in the apparatus at this moment was recorded. Next, the pressure was lowered to zero, and as the blue fluid in the cannula was forced backwards by the contents of the lymphatic sufficient pressure was again applied to bring it back to the cannula's tip. The required pressure was again recorded. The procedure was repeated several times and the average of the measurements was taken as the intra-lymphatic pressure. Finally, the pressure in the apparatus was raised slightly until the blue fluid began to move into the capillary. The pressure which was just sufficient to maintain flow was recorded. During the measurement of the pressure the ears did not alter in appearance and there was no edema visible under the microscope except in those instances of which special mention will be made later.

### *The Pressures within Lymphatic Capillaries*

The pressure in lymphatic capillaries of the ears of twenty-four mice, normal except for anesthesia and intubation, varied between zero and 2.7 cm. of water

and averaged slightly over 1.2 cm. In 80 per cent of the instances it was below 2.0 cm. In Table I the pressures are arranged in order of magnitude. In each experiment an increased pressure of 0.1 to 0.5 cm. of water produced flow into the intubated channel.

The pressure in the cutaneous lymphatic capillaries was usually lower than that in edema fluid as previously ascertained; it was even lower than many of our measurements of the normal cutaneous interstitial resistance,—which is higher than the interstitial pressure of skin (4). This finding brought up the question of whether there is a gradient of pressure existing between the inter-

TABLE I  
*Lymph Pressure as Such and the Pressure Required to  
Initiate Flow in the Lymph Capillary*

Experiment No.	Intralymphatic pressure	Pressure to yield flow	Experiment No.	Intralymphatic pressure	Pressure to yield flow
	<i>cm. of water</i>	<i>cm. of water</i>		<i>cm. of water</i>	<i>cm. of water</i>
1	0.0	0.0	13	1.2	1.6
2	0.0	0.0	14	1.3	1.5-1.6
3	0.5	0.9	15	1.4	1.8
4	0.5	0.8	16	1.5	1.7
5	0.8	1.2	17	1.5	1.7
6	0.8	1.1	18	1.5	2.0
7	0.9	1.2	19	1.8	2.2
8	1.0	1.4	20	2.0	2.4
9	1.0	1.2	21	2.0	2.5
10	1.0	1.5	22	2.1	2.3
11	1.1	1.2	23	2.7	3.1
12	1.1	1.4	24	2.7 ± 0.1	3.0
Average of all. ....				1.2-1.3	1.6

stitial fluid and the lymphatic capillaries which tends to promote lymph formation and flow. To answer this question the pressures inside and outside of the cutaneous lymphatic capillary walls were compared.

*The Relative Pressures within Cutaneous Lymphatic Capillaries and the  
Surrounding Tissues*

As has been brought out in a preceding paper (4), the true interstitial pressure in normal skin cannot be measured directly because there is not enough free interstitial fluid to allow one to make accurate manometric measurements. Nevertheless, we have estimated the true interstitial pressure closely by other means (4, 5). It was found that minute amounts of a test fluid composed of Locke's solution and 0.5 per cent of pontamine sky blue, on introduction into the skin acted as a mildly edema-forming agent, the edema increasing the bulk



of the introduced fluid very slowly. As result, for periods of 15 to 20 minutes the dye solution behaved like a relatively unabsorbable fluid. To estimate the interstitial pressure in normal skin very minute amounts of the solution were introduced into the tissues at zero pressure and in such a manner that neither blood nor lymphatic vessels were entered directly. Pressure was then gradually put upon the introduced test fluid until there occurred the slightest inward movement of it against the tissue resistance that could be measured with the techniques employed,—that is to say, an inflow averaging 0.06 c.mm. per 5 minutes. The pressure required to maintain this rate of flow was termed the “interstitial resistance” (4). It is not a measure of the interstitial pressure, for it is very slightly higher because of the pressure required to overcome the tissue resistance to the slow passage of the minute amounts of fluid employed. Both the interstitial resistance and the interstitial pressure can be measured directly (4) in edematous skin when there is free fluid present. The difference is only about 0.5 cm. of water and in normal tissues it cannot be far from this figure (4).

Since it seemed possible to learn much about the pressure conditions on both sides of the lymphatic capillary wall in normal tissues by comparing the intralymphatic capillary pressure with measurements of the interstitial resistance, experiments of the sort were carried out on the ears.

*The Determination of Interstitial Resistance with the Micro Cannula Apparatus.*—In the preceding work (4) the interstitial resistance was measured by the introduction of the mixture of dye and Locke's solution into the tissues through the smallest hypodermic needle available. For the present work the method has been simplified and improved by the use of a micro technique. After measuring the intralymphatic pressure with the micro cannula filled with the relatively unabsorbable dye-Locke's solution, the interstitial resistance was measured with the same apparatus. Under these circumstances the latter determination was accomplished with the introduction of even less fluid than was required when a hypodermic needle was employed. As result, the figures for the interstitial resistance which are here reported should lie even closer to the true interstitial pressure than those obtained in the earlier studies.

In each of eleven experiments the intralymphatic pressure was measured as described above. When several good measurements had been obtained preparations were made to determine the interstitial resistance outside of the channel by introducing the relatively unabsorbable test fluid into the tissues through the same micro cannula. While the latter remained within the channel, the pressure in the injecting apparatus was reduced to zero and at the same time the stop-cock connecting the manometers with the cannula was closed. This maneuver prevented lymph from entering the cannula as the pressure was reduced in it, and as result the cannula remained filled with the relatively unabsorbable dye-Locke's solution, undiluted by lymph, and consequently suitable for the measurement of the interstitial resistance which was to follow. Next, the mechanical stage supporting the mouse was shifted so that the lymphatic became disengaged from the cannula and the tip of the latter lay in the connective tissue close

to the channel but as far from the point of intubation as it could be moved without tearing blood capillaries. As the shift was slight, less than a quarter of millimeter, no other lymphatics were touched, and one could determine by direct observation whether or not blood capillaries were torn—in which case the experiment was abandoned. In the absence of visible injury the interstitial resistance was then measured:—

The stop-cock of the manometric apparatus was opened and a pressure of 0.5 cm. of water was put upon the fluid in the micro cannula. If no blue coloration appeared in the tissues at its tip,—and none appeared usually,—the pressure was raised to 1.0 cm. of water, and thereafter, if no flow occurred, by increments of 0.2 cm., until blue fluid began to appear in the tissues in microscopic amount. The pressure was then held at this point until one could determine whether or not the test fluid continued to enter the connective tissue and whether it entered directly into injured blood or lymphatic capillaries. If spread occurred the pressure

TABLE II  
*A Comparison of Intralymphatic Capillary Pressure and Interstitial Resistance in the Skin of the Ear*

Experiment No.	Intralymphatic pressure	Interstitial resistance	Difference
	<i>cm. of water</i>	<i>cm. of water</i>	<i>cm. of water</i>
1	Not measurable	1.5	1.5
2	0.5	1.7	1.2
3	0.7	1.3	0.6
4	0.9	1.7	0.8
5	1.0	2.1	1.1
6	1.1	1.9	0.8
7	1.5	1.5	0.0
8	1.5	1.8	0.3
9	1.8	2.5	0.7
10	2.0	2.3	0.3
11	2.6 ± 0.1	3.0	0.3-0.5
Average.....	1.2	1.9	0.7

in the apparatus was recorded as the interstitial resistance. If no spread occurred the pressure was raised by stages of 0.1 or 0.2 cm. of water until it appeared.

It is to be recalled that any mixing of the test fluid with lymph was prevented before removing the micro cannula from the lymphatic. As result the dye coloration of the test fluid remained sufficiently intense to render it visible in torn or injured blood and lymphatic capillaries. If it appeared in them the preparation was discarded. Further, the test fluid by retaining its initial composition also retained its ability to behave within the tissues like a relatively unabsorbable fluid during the period in which the measurements of interstitial resistance were made.

The findings are summarized in Table II which gives the lymph pressure within the capillaries, the interstitial resistance, and the difference between them.

In none of the eleven comparisons was the interstitial resistance lower than the lymph pressure. The latter varied more than the interstitial resistance

and the greatest differences between them were observed when the pressure in the lymphatics was low. In the first six experiments of Table II and in the ninth, the differences between the lymph pressure and the interstitial resistance were greater than 0.5 cm. of water. It seems probable that in these instances the pressure in the tissues was higher than that of the lymph within the capillaries. In Experiments 8, 10, and 11, the interstitial resistance and the lymph pressure differed by only 0.3 to 0.5 cm. of water. In all probability the pressures on both sides of the lymphatic capillary wall were approximately equal in these instances or, as in Experiment 7 in which the pressure measurements were identical, the pressure in the tissues may have been actually less than that of the lymph.

Visual observations made during these tests yielded some enlightening additional data.

In Experiment 1, Table II, the intralymphatic pressure was too low to be measured by the technique employed; the apparatus, as set up, did not permit one to measure negative pressures and at the moment the test was made the pressure may have been negative. Yet there was an obvious flow of lymph into the cannulated channel from the tributary vessel close to the tip of the micro cannula. Whenever the least pressure was put upon the contents of the cannula, blue fluid emerged from it and was gently swept away along the capillary. Evidently flow was taking place in the latter either at zero or at negative pressure. There must have been a strong pressure gradient from the tissues to the lymph.

In Experiment 7, as already mentioned, the interstitial resistance equalled the lymph pressure, indicating that the latter was higher than the true pressure in the tissues. It was noticed while measuring the pressure that the blue fluid which left the cannula proceeded in the channel only as far as the nearest valve. Just beyond this point, the lymphatic capillary passed over a vein and was squeezed shut between the vein and the skin. A pressure of 5.0 cm. of water was required to force fluid past the point of occlusion. Evidently there existed in the segment of the lymphatic capillary in which pressure had been measured a physiological blockade resulting in stagnation of lymph. This state of affairs could conceivably have produced a slightly higher pressure within the channel than in the tissues.

The manipulations involved in measuring the pressure within the capillaries often led to the appearance of edema by the time a reading of interstitial resistance could be undertaken. All instances showing frank edema under such circumstances, as manifested by a smooth and cloudy appearance of the skin or thickening of it, were excluded from Table II, but it is possible that, in the last two instances in the table, an imperceptible edema had begun to form. The lymphatic capillary pressures were higher than the average and the behavior of the fluid emerging from the micro cannula during the measurement of the interstitial resistance was not typical of that usually observed; the coloring matter forced from the cannula into the tissues spread with greater rapidity than usual. Shortly after the measurements had been made the ears became frankly edematous. In these two experiments the interstitial resistances may have been measured during an unrecognizable stage of oncoming

edema. The fact that the intralymphatic capillary pressures were high would tend to support this view. If it is correct, then in these two instances the pressures outside the capillary walls would soon have become significantly greater than the lymph pressures, judging from the other tabulated instances.

*Comparison of the Lymph Pressure and Pressure of the Fluid in Edematous Skin*

It is well known that lymph formation and flow are often greatly enhanced by the pressure of inflammatory edema (14, 15, 17-19). Further, in states of edema, free fluid is usually present in the skin and consequently the pressure existing outside the lymphatic capillaries can be accurately determined (4, 5). Accordingly edema fluid pressure was compared with that of the lymph in the capillaries.

*The Induction of Edema.*—Painting the mouse ear with xylol or heating its surface to 46°C. by means of a lamp (11) renders the skin intensely hyperemic almost at once and 5 to 15 minutes later an obvious edema appears (4, 6, 11, 20). As it develops the wrinkles of the cutaneous surface become smoother, as viewed under the microscope, and the skin itself looks clouded. Often, especially if xylol has been used, the ears assume a ground glass appearance. When pressed with a blunt instrument they "pit on pressure" or if pricked with a sharp needle droplets of fluid usually exude. When micro pipettes have already been placed in the interstitial tissues (4) to make pressure measurements, free fluid passes into the pipettes in many instances within a few minutes after the application of either heat or xylol, that is to say at about the same time that direct inspection indicates the onset of edema. Measurements of the edema fluid pressure have shown that it generally increases steadily for 2 hours or more and then slowly falls (4).

*Intralymphatic Capillary Pressure and Edema Fluid Pressure.*—In seventeen experiments lymph pressure was first measured in the lymphatic capillaries in the ears of normal mice anesthetized with nembutal in the usual way. Then, with the micro cannula still in place, a rapidly forming edema was induced by one of the methods just described. Experience gained on hundreds of edematous ears (4, 6, 11, 20) made it possible to recognize edema early, when it formed rapidly, by simple inspection under the microscope without resorting to "pitting on pressure" or other manipulations which might disturb the position of the micro cannula. At various times up to 2 hours after heating or painting the ears, that is to say during the period of rapid edema formation, the lymph pressure was again measured, often repeatedly. Just after the last measurement the stop-cock of the manometric apparatus was closed, leaving the pressure within the apparatus at the level of the intralymphatic pressure. By means of the mechanical stage the lymphatic in which pressure had just been measured was disengaged from the micro cannula, in the manner employed in the previous experiments on normal ears. A new position for the cannula was found in the connective tissue close to the channel, and the stop-cock was slowly opened. Usually colorless edema fluid began to enter the cannula, contrasting clearly with the blue fluid already there. At once the pressure in the apparatus was adjusted until the boundary of the two fluids lay at the cannula's tip. The reading then represented the edema fluid pressure. The first measurements were usually completed within 2 to 4 minutes after shifting the position of the pipette.

Table III presents the data from six experiments in which mild heat was used to produce edema. Table IV summarizes the data from eleven other experiments (Nos. 1 to 11, inclusive) in which the ear was painted with xylol.

In an additional experiment, No. 12, edema was produced 24 hours before pressure measurements were made, and consequently the lymph pressure just prior to the onset of edema is unknown.

In each of the experiments the first determination of lymph pressure, made prior to the application of heat or xylol, left a little blue fluid in the cannulated

TABLE III  
*Intralymphatic Capillary Pressure and Edema Fluid Pressure  
in Ears Subjected to Mild Heat*

Ex- peri- ment No.	Pressure before edema	Time after application of heat		Pressure during edema		Differ- ence	Remarks
	Intra- lymphatic			Intra- lymphatic	Edema fluid (intersti- tial fluid)		
	cm. of water	hrs.	min.	cm. of water	cm. of water	cm. of water	
1	0.0	0	30	2.0	5.5	3.5	
2	1.3	0	15	2.0			After the 15th minute gross swelling of the ear became visible. Now for the first time the blue fluid expelled from the micro cannula into the lymphatic while the pressure readings were taken was rapidly swept away along the channel
			20	2.6			
			32	4.0			
		1	3	3.0			
		1	6	—	6.0	3.0	
3	1.4	0	40	1.2	4.2	3.0	
4	1.5	0	20	1.5	2.2	0.7	
5	2.0	0	30	2.0	2.2	0.2	
6	2.0-2.3	0	0	2.0			Swelling of the ear appeared by the 6th minute and increased rapidly? Concurrently the blue fluid introduced into the lymphatic paled and was swept away
		0	12	1.0			
		0	21	1.5			
		0	23	—	5.0	3.5	

lymphatic. The onset of edema was frequently heralded by a rapid disappearance of the color, and through the microscope one could often observe replacement of the blue fluid by clear lymph draining into the cannulated channel through some tributary vessel. At other times the blue fluid simply paled and faded out as if dilution had taken place. As these phenomena occurred, the lymph capillary pressure rose steadily in some of the experiments and fluctuated in others. Often, as the clearance of the channels occurred, there was a temporary fall in lymph pressure.

In ten experiments, Nos. 2 and 6, Table III, and Nos. 1 to 4, 6 to 8, and 10,

TABLE IV

*The Intralymphatic Capillary Pressure and the Edema Fluid Pressure in Ears Painted with Xylol*

Experiment No.	Pressure before edema	Time after application of xylol	Pressure during edema		Difference	Remarks
	Intralymphatic		Intralymphatic	Edema fluid (interstitial fluid)		
	cm. of water	hrs. min.	cm. of water	cm. of water	cm. of water	
1	1.0	0 0	1.0			Short experiment: cannula became dislodged from the lymphatic when edema began to form. The blue test fluid introduced into the channel in taking the preliminary pressure reading was rapidly flushed away along the lymphatic when it no longer entered after the pressure in the apparatus was reduced to zero. It follows that lymph flow was taking place though the pressure was low. 45 minutes after the first edema fluid pressure measurement had been made "gel formation" occurred (see text).
		10-14	1.3-1.4			
		16	—	2.4	1.1-1.0	
		1 4	—	16.0		
2	1.5	0 5	1.5			
		11	3.3			
		27	4.8			
		46	4.8			
		48	—	7.3	2.5	
		1 35	—	7.1	—	
3	1.5	0 6	2.7			Very rapid edema formation Blue fluid in channel cleared away
		12-15	3.5			
		25	4.3			
		36	4.1			
		39	—	9.6	5.5	
		46	—	14.0	—	
	1.8	1 2	—	18.0	—	"Gel-formation"
		1 15	—	25.0	—	
		0 3	1.8			
		14	2.2			
		17	1.0			
5	1.9	23	2.0			At the 14th minute, with the pressure in the cannula at 2.2 cm. of water, blue fluid introduced into the lymphatic was rapidly flushed away although as this happened inflow from the cannula was continuing. The intralymphatic pressure fell and rose a few minutes later
		32	2.5			
		47	3.3			
		1 1	2.5			
		1 4	—	3.0-3.5	0.5-1.0	
		1 41	—	4.8	—	
		0 30	2.4 ± 0.1	2.2 ± 0.1	0.0 to -0.4	

TABLE IV—*Concluded*

Experiment No.	Pressure before edema	Time after application of xylol	Pressure during edema		Difference	Remarks
	Intralymphatic		Intralymphatic	Edema fluid (interstitial fluid)		
	cm. of water	hrs. min.	cm. of water	cm. of water	cm. of water	
6	2.0	0 3-5 7-12 30 32 45 1 12	2.2 1.5 1.5 — — —	— — 4.7-6.0 <u>17.0</u> <u>24.0</u>	3.2-4.5	At about the 6th minute after the application of xylol the blue test fluid was flushed along the channel and the pressure fell, as in Experiment 4. At the 32nd minute free fluid was still present in the tissues. At the 45th minute the ear assumed a "ground glass" appearance, the "gel-state" had appeared, and in consequence free fluid no longer entered the cannula
7	2.2	0 5 9 16 29 33 1 26	3.2 3.2 6.0 7.5 — —	— — 9.0 8.6	1.5 —	Rapidly forming edema which remained fluid
8	2.4	0 0 6 11 17 32 35 47	2.4 3.9 2.5 3.1 3.3 — —	— — — 4.1 $\pm$ 0.2 <u>19.0</u>	0.6-1.0 —	At the 5th minute intense hyperemia: the lymph pressure rose, then fell 5 minutes later and rose again at the 17th minute. At the 35th minute so little free fluid was present in the tissues that there was difficulty in obtaining the correct edema fluid pressure. At the 47th minute the "gel state" was present
9	2.5	1 4	3.8	7.0	3.2	
10	2.6	0 2 15-20 26 30-45 1 1 1 4 2 00	2.8 3.6-4.4 4.0 3.1-3.3 3.5 — —	— — — — 9.4 8.0	5.9 —	Between the 30th and 45th minutes a temporary fall in lymph pressure took place, with rapid flushing of the test fluid from the channel
11	2.8	0 5-10	3.0	3.0	0.0	Short experiment; leakage about the cannula developed at the 10th minute
12	No measurement taken prior to edema	24 0	10.0	10.0	0.0	Ear still swollen and edematous 24 hours after xylol application. Free fluid entered the cannula against a pressure of 9.6 cm. of water; lymph pressure and edema fluid pressure equal

Table IV, lymph pressure measurements were made in series as edema developed. Generally the pressure increased as time passed, but in all these instances the edema fluid pressure rose still higher. The difference in pressure between the outside and the inside of the lymphatic capillaries varied from  $-0.4$  to  $5.9$  cm. of water. This being so it is easy to see why lymph flow greatly increases in acutely edematous skin. In eight of the experiments (Nos. 1, 3 to 5, Table III, and Nos. 5, 9, 11, and 12, Table IV), the lymph and edema fluid pressures were measured but once. Pressure gradients of  $3.0$  cm. of water or more were found in eight instances (Nos. 1 to 3, 6, Table III, and Nos. 3, 6, 8, 10, Table IV) while in several others in both tables the pressure of the edema fluid approximated the lymph pressure, though it was very slightly higher. Three of the tests deserve special mention. In Experiment 5, Table IV, the edema fluid pressure was found to be equal to or  $0.3$  cm. of water lower than the lymph pressure, a difference so slight that the pressures may be regarded as equal. In two experiments, Nos. 11 and 12, Table IV, the measurements were identical. However, in one of these, No. 11, the cannula became dislodged from the lymphatic only 10 minutes after the experiment was begun and the edema had only just become visible. In the other experiment, No. 12, the measurements were made 24 hours after the application of xylol and the fully formed edema was probably receding.

*The Occasional Appearance of a Gel-Like Edema in Skin.*—A peculiar phenomenon occasionally appeared during the development of the xylol-induced edema in Experiments 1, 3, 6, and 8, Table IV. In these instances, as in all the others, the edema fluid pressure had been measured in the usual way, by first lowering the pressure within the apparatus until the edema fluid began to flow into the cannula and then finding the pressure required to stop the flow. Quite suddenly, as these measurements were repeated at time intervals ranging from 16 to 45 minutes after the application of xylol, the edema fluid failed to enter the cannula when the pressure was lowered. At first it was believed that the mouth of the cannula had become obstructed by bits of tissue or by lymph clots, and indeed this was the case in several experiments which have been ruled out. In other instances clots or obstructions were sought but not found. Since edema fluid did not enter the micro cannula, pressure was put upon its contents until the blue test fluid began to move into the tissue. To accomplish this, very high pressures,  $14.0$  to  $25.0$  cm. of water, were required, as shown by the boxed figures in column 5 of Table IV. When the positions of the micro cannulae were changed and they were inserted into the tissues at other points, the same phenomenon was encountered and no free edema fluid oozed from puncture wounds made in the skin. It was as if the free fluid had undergone the change to a gel, perhaps forming a clot as lymph often does. The phenomenon appeared only in the ears painted with xylol and may conceivably have resulted from a high degree of inflammatory irritation.



It has been mentioned earlier that the difference between the interstitial pressure and the interstitial resistance in edematous skin has been found to be approximately 0.5 cm. of water (4). In this connection it is to be noted that the high pressures shown in the boxed figures in Table IV are not to be considered as measurements of the interstitial resistance. Instead they represent the pressure necessary to disrupt the presumptive gel or clot present in those instances.

#### DISCUSSION

The pressure in the lymphatic capillaries of the mouse ear has proved to be the same or slightly less than that in the relatively large draining channels of other animals. In the cervical lymphatics of dogs lateral pressures ranged from 0.8 to 2.6 cm. of a sodium bicarbonate solution, 0.5 to 2.0 cm. of a soda solution (sp. gra. 1.080), and  $-2.8$  to  $+3.2$  cm. of water when measured by Noll (21), Weiss (22, 23), and McCarrell (24) respectively. Drinker and Field (18) found the lymph under no pressure in a resting leg of a dog, and Lee (25, 26), studying the mesenteric vessels of cats, reported pressures of 0.5 to 6.7 cm. of water in vessels proximal to the mesenteric node, and pressures of 3.0 to 6.8 in the channels distal to it. Higher pressures have been found in the larger lymphatics draining inflamed areas (17, 19) or actively moving organs (19, 24, 27), as also, of course, when end pressure measurements have been made in obstructed lymphatics (19, 24, 28), that is to say under conditions that differ widely from those considered in the present work. High pressures in minute peripheral lymphatics have been reported only by Königes and Ottó (29) who found an average pressure of 24.5 mm. of mercury in the highly specialized terminal chylous vessels of the intestinal villae of cats.

One of the early workers on the mechanism of lymph flow, Weiss (22, 23), reasoned that the pressure in lymphatics must increase in the vessels as they approach the periphery. This seemed a justifiable assumption since Rudbeck (30) had shown, as early as 1653, that ligated lymphatic vessels become distended on the peripheral side of a ligature and collapse on its proximal side. Two hundred years later Donders (31) concluded that the lymphatics would collapse if the pressures in the tissues should become higher than that in the channels. It remained for Gaskell (32) and later for Starling (33, 34) to suggest that the pressure within small lymphatics need not be higher than that in the tissues since the small lymphatics are connected to the formed elements of the connective tissues by fibrils. As result, an increase of the pressure in the tissues with consequent distention of the latter should serve to pull the lymphatic walls still farther apart rather than to collapse them. The suggestions of Starling and of Gaskell were made at a period when, as the result of writings of von Recklinghausen (35) the lymphatic capillaries were supposed to have open ends, but they have been corroborated by Clark and Clark (36), by

Pullinger and Florey (37), by work from this laboratory (7, 8, 14, 20), and by some observations made in the present studies. For example, in several of the experiments in which, after measuring the lymph pressure, edema of the ears was induced by painting the skin with xylol (Table IV) the lymphatic capillary walls suddenly became loose around the micro cannulae; as the edema developed and pressure in the tissues rose, the channels were widened and lymph began to leak out around the shafts of the cannulae, preventing further measurements of the lymph pressure. Clearly the high fluid pressure in the tissues during the early stages of edema formation did not collapse the lymphatics, but on the contrary increased their diameter, and under the circumstances it must have acted to force fluid through the capillary walls to form more lymph.

The existence of an effective pressure difference between the edema fluid and the capillary lymph, as shown in the present work, not only discloses a mechanism which will account at least partially for the flow of lymph but it furnishes evidence for the generally accepted view that the lymph capillaries have walls devoid of fenestrations. This evidence corroborates the anatomical studies of others (38-42). It also confirms work from this laboratory (7, 10, 11, 20) in which the behavior of dyes injected into lymphatic capillaries indicated an unbroken continuity of the vessel walls.

Despite their continuity the walls of the lymphatic capillaries are extremely permeable, even more so than blood capillary walls. This is evident in the fact that dyes escape from lymphatic capillaries into the surrounding tissue about as fast as from blood capillaries, although the hydrostatic pressure existing in the lymphatic channels and tending to force fluid through their walls is far lower than in the capillaries carrying blood (10, 11). Since tissue fluid gets into the lymphatics so readily, and lymph flow is relatively slow, what can be responsible for the retention of the fluid in the lymph capillaries until it reaches the larger, relatively thick-walled channels from which it cannot escape? It seems reasonable to suppose that the retention is due to the pressure of the interstitial tissue fluid. Undoubtedly this is the case in the skin of the ears of mice during the rapid formation of edema, but does it hold as well in normal ears in which the true interstitial pressure cannot be measured? As has already been brought out above, the interstitial resistance, measured in the present work by micro methods, must have been very close to the true interstitial pressure. The average of all the measurements of interstitial resistance shown in Table II was 1.9 cm. of water as compared with 1.2 for the lymph pressure. In more than half the experiments on normal ears there must have been a gradient of pressure from the tissues to the lymph, for the interstitial resistance was higher than the lymph pressure by more than 0.6 cm. of water. In the remaining tests the interstitial pressure and the lymph pressure, as judged by measurements of the interstitial resistance, were probably

about equal. Under these circumstances there was little reason for the fluid in the lymphatic capillaries to escape before an alteration in local conditions caused it be moved into the thick-walled drainage channels.

#### SUMMARY

The pressure in the cutaneous lymphatic capillaries of normal mice anesthetized with nembutal ranged between 0.0 and 2.7 cm. of water. Measurements of the interstitial pressure in the tissue immediately next the lymphatics showed that, in more than half the instances studied, there was a slight gradient of pressure from the tissues to the lymph. In nearly all the other instances the pressures inside and outside the lymphatic capillaries were approximately equal. In two cases in which lymph flow in the capillaries was rapid, the lymph pressure may have been negative. Under these circumstances there must have been a considerable gradient of pressure from the tissues to the lymph.

In skin which was rapidly becoming, or had recently become, edematous as result of the application of xylol or of heat, the intralymphatic capillary pressure generally was increased, yet when compared with the pressure prevailing in the edema fluid outside of the capillaries it was usually found to be relatively much lower, at times by as much as 5.9 cm. of water. The findings indicate that a pressure gradient is an important factor in lymph formation under normal and pathological circumstances.

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# THE SUSCEPTIBILITY OF INFANT RHESUS MONKEYS TO POLIOMYELITIS VIRUS ADMINISTERED BY MOUTH

## A STUDY OF THE DISTRIBUTION OF VIRUS IN THE TISSUES OF ORALLY INFECTED ANIMALS\*

BY DOROTHY M. HORSTMANN, M.D., JOSEPH L. MELNICK,<sup>†</sup>PH.D.,  
ROBERT WARD, M.D., AND M. JOSÉ SÁ FLEITAS, M.D.†

(From the Section of Preventive Medicine of Yale University School of Medicine,  
New Haven)

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The experiments to be described were undertaken with a twofold purpose: (1) to test the susceptibility of *infant rhesus* monkeys to poliomyelitis virus fed by natural means; and (2), to study the distribution of virus in the tissues of this species when infected by the oral route and to compare the pattern obtained with that reported for *cynomolgus* monkeys infected orally (1, 2) and for fatal human cases (3-10).

*Earlier Feeding Experiments. The Susceptibility of Various Monkey Species.*—The earliest successful attempt to produce infection by the gastrointestinal route is that reported by Leiner and von Wiesner in 1910 (11). These workers fed large amounts of virus by stomach tube to two monkeys (species not mentioned), one of which developed paralytic poliomyelitis, confirmed by histological sections. They also inoculated virus directly into the ileum at laparotomy and produced "characteristic symptoms" in three of four animals tested. Between the years 1910-1929, attempts to repeat the work of Leiner and von Wiesner were largely unsuccessful. Flexner and Lewis (12), Landsteiner, Levaditi, and Pastia (13), Amoss (14), Rhoads (15), Schultz (16), and Thompson (17) all described negative results.

Although various monkey species and strains of virus were used in these experiments there was no attempt to evaluate such variables until the work of Kling, Levaditi, and their associates Lépine and Hornus (18-21). These investigators, between 1929 and 1934, reported a series of experiments testing the susceptibility of both *rhesus* and *cynomolgus* monkeys to poliomyelitis virus fed orally, injected by stomach tube, and introduced directly into the intestinal tract at laparotomy. They were able to infect nine of twelve *cynomolgus*, but failed when *rhesus* monkeys were used. Burnet and his coworkers confirmed these observations in 1939 (22): eight of fifteen *cynomolgus* to which virus was administered orally, by pharyngeal swab, or by stomach tube, developed poliomyelitis. (An objection had been raised to the earlier successful experiments of Kling and his group, on the ground that when infection was produced by the oral route virus may have entered through the olfactory

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† Alfredo Hirsch Foundation Fellow of the Academia Nacional de Medicina, Buenos Aires, Argentina.

pathway. This difficulty was anticipated by Burnet and his colleagues who blocked the nasal pathway in some of their animals by preliminary application of a  $\text{ZnSO}_4$  spray to the nasal mucosa.) Subsequently, *cynomolgus* monkeys have been infected a number of times by oral administration of virus (1, 23, 24) and other species a few times: *M. irus (mordax)*<sup>1</sup> in one of eleven trials (23, 26) the green African monkey, *Cercopithecus aethiops sabaeus*, in one of seventeen (25, 26). Negative results have been obtained with one capuchin (*Cebus fatuellus*) (25), one grivet (*Cercopithecus griseoviridis*), (28), seven vervets, *Cercopithecus aethiops pygerythrus*, (27, 28), two *M. sinicus* (29, 30), and two baboons one of which was of the species *Papio hamadryas* (27, 31).

The experimental animal which has proved most susceptible to infection by the gastrointestinal or oral route of virus administration is the chimpanzee. But it was many years before this was appreciated. Landsteiner and Levaditi first attempted such an experiment in 1910 (30) by feeding a chimpanzee 10 cc. of infected monkey cord by stomach tube. The animal remained well and the result was interpreted as negative. However, since it is now known that in chimpanzees infection is often manifested only by a "healthy" carrier state for a period of weeks (33, 34, 35) it is possible that the animal used by Landsteiner and Levaditi did become infected even though it never developed paralysis. Chimpanzees were not used for similar experiments until 1942 when Howe and Bodian induced poliomyelitis in five of five animals using infective human stools as the inoculum, which was administered by simple feeding, by stomach tube, and by lingual and buccal swab (31). The olfactory tracts of the animals were sectioned before the experiment was begun in order to block the olfactory pathway. Subsequently, a "healthy" carrier state was produced in each of thirteen chimpanzees tested in Baltimore (33) and in eight chimpanzees in New Haven (34, 35).

The results with chimpanzees are in marked contrast to the experience with *rhesus* monkeys. In the latter, of the twenty-three reported animals in experiments in which it has been specifically stated that *M. mulatta* were used for various types of feeding experiments (virus being given by mouth, by stomach tube, and rectal tube) twenty-three negative results have been reported; of these, twenty-two attempts were in young or adult *rhesus*, while one was in a 3 month old baby *rhesus* (31). Leiner and von Wiesner (11) reported a single positive, although it is not entirely certain that the species used was *M. mulatta*. In recent unpublished experiments from our laboratory, one young *rhesus* of eight fed the Y-SK strain developed paralytic poliomyelitis (32); previous experience with feeding this strain of virus to young adult *rhesus* monkeys had been negative (23).

*Strains of Virus.*—The experiments of Sabin (1) indicate the possible rôle of virus strain differences in producing infection by the oral route. Six of fifteen *cynomolgus* monkeys developed paralyzes when fed the recently isolated "Per" strain, while none of five *cynomolgus* showed signs of disease when fed the Rockefeller MV strain. Earlier experiments of Clark and his associates (36, 46) and Flexner (5) in which both *cynomolgus* and *rhesus* were fed the highly monkey-adapted MV strain, were

<sup>1</sup> There are at least several branches of the *cynomolgus* family, and the terminology is not perfectly clear. Those which have been used include, *M. cynomolgus* (Java), *M. irus (mordax)*, *M. filipinensis* (Philippine Islands), and *M. irus valida* (Siam).

also negative. Burnet and his group used recently isolated human strains in their successful feeding experiments (22), as did Vignec, Trask, and Paul (23) who used early passages of the Y-SK strain. The successful tests in chimpanzees have been with human stool suspensions (31), fly-contaminated food (34), and the Y-SK strain (35).

*Site of Penetration.*—In attempting to localize the level of the gastrointestinal tract at which the virus penetrates the intact mucous membrane, Faber, Silverberg, and Dong (24) fed virus ("Per" strain) to *cynomolgus* in capsules, applied it by lingual swab, and injected it by enema. Positive results were obtained only with the lingual swab technique in one of eighteen trials.

*Summary.*—Table I summarizes the reported experiments dealing with the production of poliomyelitis by various oral and gastrointestinal routes. The number of uncontrolled variables in these experiments make it impossible to compare the susceptibility of the different species more directly. Although such a tabulation would make for simplicity and clarity, it would nevertheless be misleading. The table is therefore constructed on chronological lines.

### *I. The Susceptibility of Infant Rhesus Monkeys to Orally Administered Poliomyelitis Virus*

In view of the apparent resistance of young or adult *rhesus* monkeys to orally administered poliomyelitis virus, the following experiments were planned to test the susceptibility of *infant rhesus* monkeys to poliomyelitis virus administered orally. An attempt was made to reproduce as far as possible a "natural" mode of infection, and simple virus feeding alone was therefore employed. In order to avoid the possibility of infection by the olfactory pathway, the nasal mucosa of each animal was sprayed vigorously with 1 per cent  $\text{ZnSO}_4$  on two occasions 2 to 5 days apart, before feeding was begun. This procedure has been shown by Schultz and Gebhardt (37) to block the penetration of virus through the nasal mucosa to the olfactory tract.

### *Materials and Methods*

*Monkeys.*—Seven infant *rhesus* monkeys, *M. mulatta*, ranging in age from 3½ weeks to approximately 4 months and one young adult *rhesus* were used (see Table II).<sup>2</sup> During the course of the experiments each animal was observed daily for signs of poliomyelitis, and daily temperature records were kept. Those animals developing fever and paralysis were sacrificed early in the disease in the hope of obtaining a maximum yield of virus from the various tissues. Animals showing no signs of disease were observed for 5 to 6 weeks; they were sacrificed at the end of this period, and sections of their olfactory bulbs, brain stem, and cervical, thoracic, and lumbar cords were made for histological examination.

*Virus Strains.*—A variety of strains were used. They included two rodent-adapted strains (Y-SK and Ph), infective human CNS and stools, and monkey cord infected with recently isolated human strains. Table II indicates the various strains fed to each animal. Some

<sup>2</sup> We are indebted to Dr. G. van Wageningen of the Department of Obstetrics and Gynecology, Yale School of Medicine, for most of the infant animals used in these experiments.

TABLE I

*Attempts to Produce Experimental Poliomyelitis by the Oral and Gastrointestinal Routes*

Year	Authors	Animal species	Inoculum	Re-sults	Comment
1909	Landsteiner and Levaditi (29)	<i>M. sinicus</i>	Virus emulsion by stomach tube	0/1	
1910	Leiner and von Wiesner (11)	Not given	(a) 80 cc. of cord emulsion by stomach tube, 2-3 times (b) Virus inoculated directly into ileum at laparotomy	1/2 3/4	Histology +; O.B. n.ex. All animals received morphine before inoculation to reduce intestinal motility
1910	Landsteiner and Levaditi (30)	Chimpanzee	10 cc. cord suspension by stomach tube	0/1	
1911	Landsteiner, Levaditi, and Pastia (3)	<i>M. sinicus</i>	Cord suspension in milk by stomach tube-340 cc. over 17 days	0/1	
		<i>M. mulatta</i>	Cord suspension in milk by rectal tube-130 cc. over 26 days	0/1	
1929	Rhoads (cited by Flexner (5))	<i>M. cynomolgus</i>	12-25 cc. 20 per cent "mixed virus suspension"	0/3	Preliminary treatment with NaHCO <sub>3</sub> and opium
1929	Schultz (16)	Not given	Aycock-Flexner strain-7 gm. monkey cord in 150 cc. milk, 3 times in 24 hours	0/2	
1930	Demme (42)	Not given	Kling strain-passaged in monkeys for 15 years. Cord suspension by stomach tube	1/2	Positive monkey received preliminary treatment with sapoin to produce diarrhea
1930	Thompson (17)	<i>M. mulatta</i>	Aycock strain-20 per cent suspension 10-15 cc. by stomach tube 2-4 times over 18-51 days	0/4	Preliminary treatment with bile as irritant
1930	Clark, Schindler, and Roberts (36)	Not given	20-25cc. cord suspension every 5 days for 8-14 weeks	0/3	
1929	Kling, Levaditi, and Lépine (18)	<i>M. cynomolgus</i>	Cord emulsion. 10-35 cc. 1-3 doses (2 by stomach tube, 1 by mouth)	3/3	Histology +; O.B.n. ex
		<i>M. cynomolgus</i>	Cord suspension-1 cc. directly into ileum at laparotomy	1/1	Histology +; O.B.n.ex.
		<i>M. mulatta</i>	Cord suspension-45 cc. in 3 doses by mouth	0/2	
1931	Levaditi, Kling, and Lépine (19)	<i>M. cynomolgus</i>	Cord suspension in potato, banana by mouth	1/1	Histology +; O.B.n.ex
		<i>M. cynomolgus</i>	Cord suspension by stomach tube. 10-25 cc., 1-2 doses	3/5	Histology +; O.B. n.ex.
1933	Levaditi, Kling, and Hornus (20)	<i>M. mulatta</i>	20 per cent cord suspension by stomach tube daily for 3 days	0/1	
		<i>M. cynomolgus</i>	Fed virus in butter daily 5-10 doses	1/2	Histology +; O.B. n.ex.



TABLE I—Continued

Year	Authors	Animal species	Inoculum	Results	Comment
1934	Kling, Levaditi, and Hornus (21)	<i>M. cynomolgus</i>	Virus injected directly into intestine 5-10 cc. doses	4/16	Histology +; O.B. n.ex.
1932	Saddington (43)	<i>M. cynomolgus</i>	Cord suspension in milk 180 cc. over 6 days by dropper	1/1	Histology +; O.B. n.ex.
1932	Clark, Roberts, and Preston (46)	<i>M. cynomolgus</i>	Rockefeller MV strain 25-60 cc. 5 per cent cord suspension by stomach tube over 2 days	0/2	
		<i>M. cynomolgus</i>	10 cc. MV cord suspension directly into ileum, duodenum	0/3	
1936	Flexner (5)	<i>M. cynomolgus</i>	10 per cent MV suspension 180-410 cc. in 7 feedings by stomach tube	0/3	
		<i>M. cynomolgus</i>	180-210 cc. of same material by dropper in 7 feedings	0/2	
		<i>M. cynomolgus</i>	25 per cent MV and Phil., 1932, cord 35-45 cc. in 2-4 feedings by stomach tube	0/2	
		<i>M. cynomolgus</i>	25 per cent MV and Phil., 1932, cord into intestinal tract at laparotomy	2/5	Histology + in animal sacrificed. O.B. —
		<i>M. mulatta</i>	Same	0/2	
1936	Lennette and Hudson (44)	<i>M. mulatta</i>	Suspensions of cord directly into isolated loops of bowel every 3-7 days for 3 mos.	0/4	
1939	Burnet, Jackson, and Robertson (22)	<i>M. cynomolgus</i>	Thick suspension of monkey cord "Mar" strain		
			(1) Fed 1 cc. with pipette	2/6	
			(2) Inoculated into stomach	0/1	
			(3) Inoculated into duodenum	1/1	
			(4) Swabbed tonsillar region	1/2	Olfactory bulbs of +monkey negative on subinoculation
			(5) Same	2/4	Preliminary ZnSO <sub>4</sub> to nasal mucosa. Histology +; O.B. n.ex.
			(6) Swabbed tongue	2/4	Histology +; O.B. n.ex.
1939	Vigne, Trask, and Paul (23)	<i>M. mordax</i>	0.9 gm. early passage Y-SK strain cord in banana over 3 days	1/9	Histology +; O.B. —
		<i>M. cynomolgus</i>	3 gm. Y-SK cord in banana	1/1	Histology +; O.B. +
		<i>M. mulatta</i>	Y-SK in banana	0/8	
1940	Rasmussen and Clark (45)	Not given	25 cc. virus suspension by stomach tube daily for 3 days	0/2	Preliminary treatment with starch intracerebrally
1941	Trask and Paul (25)	<i>C. fauvelus</i>	Y-SK monkey cord suspension in banana	0/1	
		<i>M. mordax</i>	Same	0/2	
		<i>M. cynomolgus</i>	Same	0/1	
		<i>C. adhiops sabens</i>	Same	1/7	Histology +; O.B. —

TABLE I—Continued

Year	Authors	Animal species	Inoculum	Re- sults	Comment
1942	Howe and Bodian (31)	<i>C. aethiops</i> <i>pygerythrus</i> (vervet)	Pooled positive human stools by stomach tube	0/1	Paralytic poliomyelitis developed in all 5 animals. Olfactory tracts sectioned be- fore feeding begun
		Baboon	Same	0/1	
		<i>M. cynomol-</i> <i>gus</i>	Same	0/2	
		<i>M. mulatta</i> aged 3 mos.	Same	0/1	
		<i>M. mulatta</i>	Same (by rectal instead of stom- ach tube)	0/6	
		Chimpanzee ( <i>Pan saty-</i> <i>rus</i> )	Pooled human stools orally	2/2	
			Same—by stomach tube	2/2	
1944	Sabin and Ward (1)		Same—swabbed on tongue and lips	1/1	Histology +; O.B. + in 2/6
		<i>M. cynomol-</i> <i>gus</i>	10–20 cc. "Per" strain cord sus- pension in banana	6/15	
		<i>M. cynomol-</i> <i>gus</i>	Same—but using MV strain	0/5	
1943	Faber, Silverberg, and Dong (24)	<i>M. cynomol-</i> <i>gus</i>	0.6 gm. Per strain cord, dried, given in capsule	0/26	Negative animals used repeatedly Histology +; O.B. —
			Same material swabbed on tongue	1/13	
			Same—by enema	0/11	
1944	Paul (27)	<i>C. griseoviri-</i> <i>dis</i> (grivet)	1–5 cc. 10 per cent infected hu- man spinal cord by stomach tube	0/1	
		<i>C. aethiops</i> <i>pygerythrus</i> (vervet)	Same	0/1	
		<i>Papio hama-</i> <i>dryas</i> (ba- boon)	Same	0/1	
1944	Melnick*	<i>C. aethiops</i> <i>pygerythrus</i> (vervet)	10–20 per cent Y-SK cotton rat cord 2 cc. daily for 8 days	0/5	Preliminary ZnSO <sub>4</sub> to nasal mucosa
1945	Howe and Bodian (33)	Chimpanzee	Positive human stool suspension by stomach tube, swabbed on tongue and buccal surfaces	6/6	All animals became in- testinal carriers of virus; none devel- oped paralytic dis- ease. Histology +. O.B. + in 2 of 6
		Chimpanzee	16–18 cc. positive human stool by mouth	2/2	Both became intestinal carriers with no pa- ralysis. O.B. +
		Chimpanzee	20 cc. stool by mouth	21/1	Neither carrier state nor paralysis. Au- topsy after 7½ mos. showed minimal lesions

TABLE I—*Concluded*

Year	Authors	Animal species	Inoculum	Re- sults	Comment
1945	Ward, Melnick, and Horstmann (34)	Chimpanzee	North Carolina, 1944, fly con- taminated food	2/2	All animals became in- testinal carriers of vi- rus; none developed paralytic disease
1947	Melnick and Horst- mann (35)	Chimpanzee	Y-SK strain 10 per cent cotton rat cord by mouth	3/3	
1947	Howe and Bodian (26)	<i>C. aethiops</i> <i>sabaeus</i>	5 cc. 20 per cent emulsion cord— "Brunhilde II virus pool"	0/10	Stools of all animals, collected between 14 and 16 days after feeding, negative in <i>rhesus</i> monkeys

Results of histological examination of olfactory bulbs recorded as follows: O.B. + (positive); O.B. - (negative); O.B. n.ex. (not examined).

\* Unpublished data.

animals received one form of virus suspension only, while others were given several types of material sometimes in pools of each.

**Administration of Virus.**—Five of the animals were fed virus suspended in a milk formula in ordinary baby nursing bottles with rubber nipples. The formula, bottles, and nipples, were all sterilized before each use. Two animals were fed virus in milk, drop by drop, from a sterile pipette. In several, the milk feeding was supplemented by virus suspension inoculated into bananas. Some of the stools were fed as crude suspensions; others were first concentrated by ultracentrifugation (38). The CNS materials were given as 10 to 20 per cent suspensions. The number of feedings varied from one to seventeen per monkey, and the dosages from 6.6 cc. of ultracentrifuged concentrate to 273 cc. of 10 per cent suspension. (See Table II.)

## RESULTS

Two of the seven baby *rhesus* developed paralytic poliomyelitis after being fed virus.

One animal, Rh. 27-06, developed fever, irritability, and questionable weakness of the legs on the 8th day after feeding was begun, and complete paralysis of the legs on the 9th day (Fig. 1). The total virus dosage was 53 cc. of a 10 per cent suspension of Ph strain, the last 10 cc. of which were given early on the day symptoms first appeared. Histological sections revealed typical lesions of poliomyelitis in the cervical, thoracic, and lumbar cord, consisting of marked perivascular infiltration and neuronophagia. The olfactory bulbs were negative when examined in serial sections.

The other positive result was obtained with Rh. 27-38. This animal had received a total of 95 cc. of Y-SK strain, consisting of 10 per cent suspension of cotton rat and monkey cord. Feeding was carried out over a period of 12 days (Fig. 1). Fever, irritability, and ear tremor developed 17 days after feeding was begun, and 6 days after the last dose of virus had been administered. On the 18th day, the signs were more marked and the legs seemed weak. On the 19th day, the temperature fell and there was no further progression of weakness. The animal was sacrificed on this day. Histological sections of the medulla, cervical, thoracic, and lumbar cord revealed typical lesions of poliomyelitis. Serial sections of the olfactory bulbs were negative as were sections of the gasserian ganglia.

A third monkey, Rh. 27-05, had an episode of fever, anorexia, and irritability lasting 3

TABLE II  
*Susceptibility of Infant Rhesus Monkeys to Poliomyelitis Virus  
 Administered by the Oral Route*

Monkey No.	Age of monkey	Strain fed	Inoculum	Amount	Dates of feeding	Results	
						Paralysis	Spinal cord lesions
27-06	3½ wks.	Ph (Middle East)	10 per cent mouse CNS	53 cc.	1944 4/14-4/22	+	+
27-38	3½ wks.	Y-SK	10 per cent cotton rat CNS	20 cc.	5/29-5/30		
			20 per cent monkey cord	75 cc.	5/31-6/9	+	+
27-31	4 wks.	Mixed—many U. S. strains	Pool of ultracentrifuged positive human stools	6.6 cc.	5/13	—	
		Chicago, 1943 and New Haven, 1944	10 per cent and 20 per cent human cord	293 cc.	7/28-8/17	—	—
27-05	10 wks.	Chicago, 1943	Pool of positive human stools	73 cc.	4/14-4/23		
			Ultracentrifuged concentrate stool suspension	8 cc.	4/25	—	—
27-32	3 mos. ±	Ph (Middle East)	10 per cent mouse CNS	19 cc.	5/19-5/20	—	
			10 per cent mouse CNS	20 cc.	7/28-7/30	—	—
31-17	4 mos. ±	North Carolina, 1944	20 per cent monkey cord	77 cc.	8/10-8/24	—	—
31-18	3 mos. ±	North Carolina, 1944	Ultracentrifuged concentrate positive human stools	28 cc.	8/10-8/15		
		New York City, 1944	Sharples concentrate positive human stools	28 cc.	8/20-8/25	—	—
27-23 (control)	2 yrs. ±	Ph (Middle East)	10 per cent mouse brain	40 cc.	5/20-5/28	—	—
			Mouse brains	0.8 gm.		—	—

days which was thought to be compatible with abortive poliomyelitis. This animal had received a total of 73 cc. of crude stool suspension and 8 cc. of ultracentrifuged concentrate of fecal material. The onset of symptoms was 15 days after virus feeding was begun and 3 days after it had been stopped. Stools collected on the 3rd day of illness and during the subsequent 8 days were pooled, concentrated by ultracentrifugation, and tested intracerebrally in another *rhesus* monkey with negative results. Rh. 27-05 was sacrificed 24 days after this mild illness (41 days after the feedings was begun). No lesions were found in three levels of the cord or in the medulla or olfactory bulbs.

The other four animals, as well as the control, remained entirely well during the experiment. Two animals (27-31, 27-32) had been given small doses of virus and observed for the usual period for signs of disease. Both remained healthy and after 5 weeks were fed the same or different strains in large amounts, again with negative results (Table II).

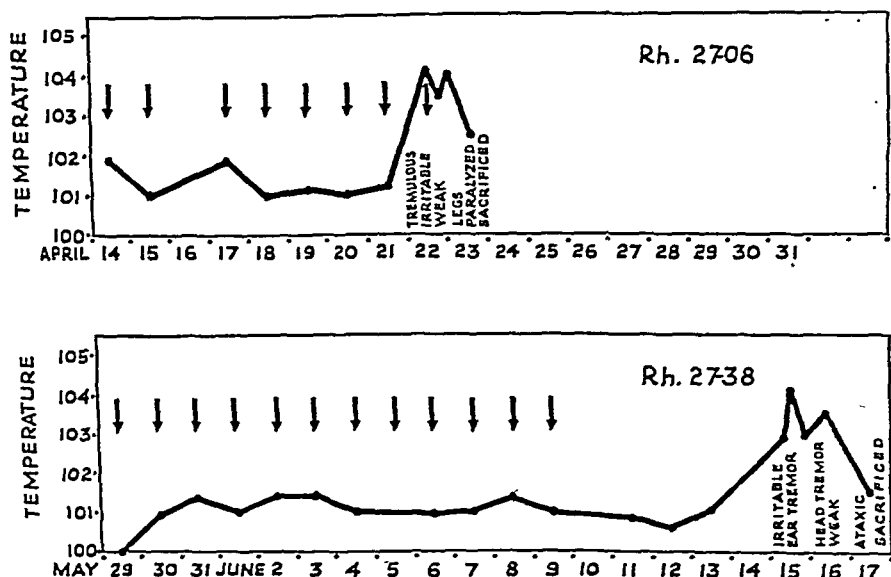


FIG. 1. Course of events in two baby *rhesus* infected with poliomyelitis virus by the oral route. Arrows indicate times at which virus was fed.

## II. Distribution of Poliomyelitis Virus in the Tissues of Two Infant *Rhesus* Monkeys Infected by the Oral Route

Studies on the localization of poliomyelitis virus in the fatal human disease (3-10) and in *cynomolgus* monkeys infected by the oral route (1, 2) have shown different distribution patterns. While in neither case does the virus seem to be as strictly neurotropic in its location as was once believed, it is more diffusely distributed throughout the tissues of the *cynomolgus* monkey than in man. In their series of human autopsies on five bulbar and two spinobulbar types of cases Sabin and Ward (8) found virus primarily in the nervous system, the alimentary tract (including the pharynx), and superficial lymph nodes. The results in less extensive studies of human tissues have been similar (3-7, 9, 10).

In studies using *cynomolgus*, Sabin and Ward found that not only these systems, but blood, trachea, liver, spleen, kidney, and bladder also yielded positive tests for virus (1). In the present study, an attempt was made to determine the virus distribution in orally infected *rhesus* monkeys and to compare it with the pattern found in the human disease and in orally infected *cynomolgus* monkeys.

### *Material and Methods*

*Monkey Tissues.*—The two infant *rhesus* 27-06 and 27-38 which developed paralytic poliomyelitis after being fed large amounts of virus were sacrificed as soon as the diagnosis became clear. The animals were killed under ether anesthesia by bleeding from the heart. Autopsies were performed using sterile technique. Various tissues were removed separately, each with a different set of sterile instruments. The olfactory bulbs were fixed whole in formalin for serial sectioning. The other tissues were frozen on dry ice in individual sterile lusteroid tubes.

*Tests for Virus.*—The testing for virus was carried out in young *rhesus* monkeys, mice, and cotton rats by intracerebral inoculation of the various tissue extracts. The use of rodents was possible because both animals had been infected with rodent-adapted strains of virus. Lymph nodes, spinal cord, and adrenals were inoculated as 20 per cent suspensions. The rest of the tissues were extracted with  $H_2O$  and the extracts concentrated by ultracentrifugation (38). The test monkeys were observed daily for 4 to 6 weeks before being sacrificed for histology, and the negative rodents were discarded at the end of the observation period. Criteria for a positive test were: the development of typical signs of the disease during life with characteristic histological lesions in the monkey, and the development of signs of the disease only in rodents. Passage to other rodents and to monkeys was carried out in some instances.

### *Results of the Study of Virus Distribution*

A summary of the tests for virus is presented in Table III. From Rh. 27-06, fed Ph strain and killed on the 2nd day of its disease, virus was recovered from spinal cord, superficial lymph nodes, buccal mucosa, small intestinal wall, colon contents, heart muscle, spleen, and adrenal glands. Serial sections of the olfactory bulbs showed no lesions. No virus was found in the blood, pharyngeal wall, mesenteric lymph nodes, celiac plexus, liver, kidney, or lungs. The spinal cord was passed successfully to a monkey, mice, and cotton rats. Brain passage of mice and cotton rats paralyzed after receiving suspensions of heart and adrenal gland was carried out in mice, cotton rats, and *rhesus* monkeys (Fig. 2). Passage was successful in all instances. That the virus detected in mice was the same as the one fed to Rh. 27-06 (and not one indigenous to mice, *viz.* Theiler's) was evident from the fact that the mouse passage material retained its monkey pathogenic property.

Rh. 27-38, following the ingestion of the Y-SK strain in large amounts, was killed on the 3rd day after its first symptoms of poliomyelitis. Although extensive lesions were present in the spinal cord and medulla, we were unable to recover virus from any of the tissues of this baby monkey. In addition, stools, collected during the 2nd and 3rd days of virus feeding (14 days before onset of symptoms) were pooled, ultracentrifuged, and tested in rodents. No

TABLE III

Distribution of Virus in the Tissues of Infant Rhesus Monkeys Infected by the Oral Route

Tissue	Rh 27-06 (José)			Rh. 27-38 (Francisco)		
	Mice	Cotton rats	Monkeys	Mice	Cotton rats	Monkeys
Heart blood.....	0/8*	0/9		0/12	0/6	
Tongue.....	0/12	0/5		0/9	0/5	
Buccal mucosa.....	1/11	0/6		0/11	0/6	
Pharyngeal wall.....	0/11	0/6		0/10	0/5	
Axillary lymph nodes.....	2/10	4/7		0/10	0/3	
Inguinal lymph nodes.....				0/13	0/3	
Cervical lymph nodes.....	1/8	—		0/12	0/6	
Submental lymph nodes.....				0/12	0/6	
Mesenteric lymph nodes.....	0/10	0/9		0/13	0/3	
Celiac plexus.....	—	0/5		0/12	0/3	
Spleen.....	0/12	1/4		0/12	0/6	
Kidney.....	0/11	0/6		0/12	0/3	
Adrenal.....	0/12	3/6		0/11	0/6	
Liver.....	0/12	0/5		0/11	0/6	
Small intestinal contents.....	0/10	0/10		0/4	0/5	0/1
Small intestinal wall.....	3/8	0/11		0/10	0/4	0/1
Colon contents.....	4/8	2/9		0/5	0/5	0/1
Colon wall.....	0/9	0/10		0/10	0/4	0/1
Lung.....	0/11	0/4		0/7	0/6	
Heart muscle.....	3/12	6/6		0/4	0/4	
Anterior perforated substance.....	0/4	0/9		—	—	
Nasal mucosa and nasal bones.....	0/12	0/6		0/12	0/6	
Spinal cord.....	3/10	3/4	1/1	0/19	0/10	0/4

\* Numerator = number of test animals which developed poliomyelitis. Denominator = number of animals on each test.

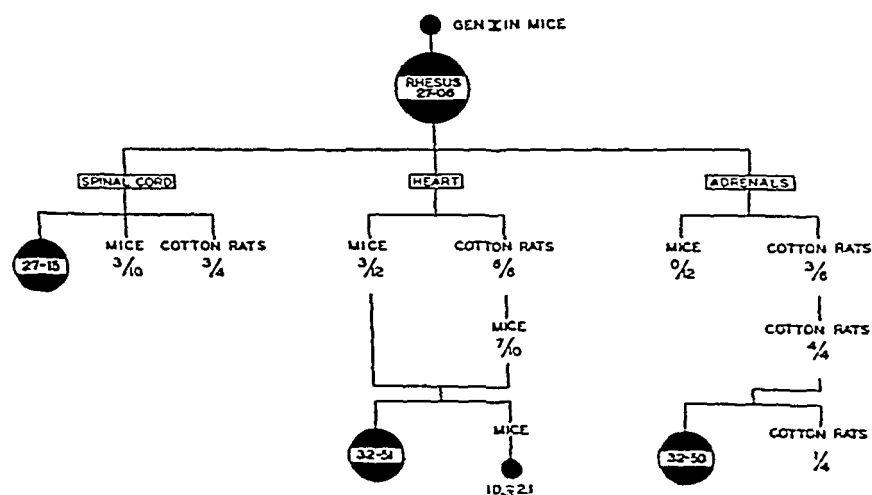


FIG. 2. Passage of tissues of infant rhesus (27-06) to monkeys, mice, and cotton rats. 3/10 = three mice paralyzed of ten mice inoculated. Large black circles = rhesus monkeys paralyzed by intracerebral inoculation.

virus was recovered. The spinal cord was tested as a 10 per cent suspension in two monkeys with negative results. The cord suspension was then concentrated by ultracentrifugation and tested in twelve mice, ten cotton rats, and two more monkeys, again with entirely negative results. Ultracentrifuged concentrates of colon contents, colon wall, and small intestinal contents and wall were all negative in monkeys as well as in rodents.

#### DISCUSSION

It has been demonstrated in these experiments that baby *rhesus* monkeys can occasionally be infected with poliomyelitis virus by the oral route. In our tests there were two positive results of seven trials. From this small number questions of strain difference, age of the monkey, and dosage cannot be evaluated in the susceptibility of infant *rhesus* to orally administered virus. But the enormous doses of virus used to transmit the disease and the failure of some animals to become infected on even larger doses, suggest that even the infant *rhesus* is relatively resistant to virus administered by this peripheral route. Both of the strains which produced infection are rodent-adapted ones related serologically to the Lansing strain (39). A variety of human stool and central nervous system strains either as fresh human material or as early monkey passage CNS failed to produce infection. However, the two positive animals were both 3 to 4 weeks old, whereas three of the four negative ones were  $2\frac{1}{2}$  to 4 months old, and only one was 4 weeks of age.

The recovery of virus from the tissues of one baby monkey paralyzed after being fed virus, and failure to recover it from another similarly infected is difficult to understand. Both animals were killed early in the course of disease (48 and 72 hours after onset) and both showed extensive spinal cord lesions histologically. Rh. 27-06, whose tissues were positive for virus, was fed infective material the day symptoms first appeared, 24 hours before being sacrificed. Although it is possible that virus isolated from this animal's buccal mucosa, and perhaps the cervical and submental lymph nodes, may have represented the inoculum of virus which had been ingested 24 hours previously, it seems unlikely that this was the whole explanation for the positive tests for virus isolated from the axillary and inguinal nodes, CNS, or other tissues. The animal whose tissues were negative, had not been fed virus for 6 days before onset of symptoms. The failure to recover virus even from the cord of this second animal, despite the relatively short time between onset and autopsy, and the extensive CNS lesions, is perhaps comparable to the occasional negative tests obtained with human cords of patients dying in the acute stage of the disease.

Although it would be unwise to generalize on the anatomical pattern of distribution of virus from one orally infected *rhesus* monkey, this pattern appears to be less diffuse than that found by Sabin and Ward (1) for the *cynomolgus* monkey and closer to that reported for the human disease (see Table IV).



Yet virus was isolated in the infant *rhesus* from the heart and adrenals—the latter having been negative in the case of human autopsies and *cynomolgus*

TABLE IV

*Comparison of Virus Distribution in Orally Infected Rhesus and Cynomolgus Monkeys and Human Tissues at Necropsy*

Tissue	Infant* <i>rhesus</i>	<i>Cynomolgus</i> †	Human beings‡
Spinal cord.....	1/2	5/5	25/46
Olfactory bulbs.....	0/2	1/6	1/17
Nasal mucosa.....	0/2	1/4	1/9
Buccal tissue.....	1/2	4/5	0/6
Tongue.....	0/2	4/5	1/6
Pharyngeal wall and tonsils.....	0/2	5/8	14/36
Small intestinal wall.....	1/2	2/5	6/15
Small intestinal contents.....	0/2	—	3/13
Colon contents.....	1/2	3/3	15/30
Colon wall.....	0/2	1/5	1/14
Celiac plexus.....	0/2	1/7	1/9
Heart.....	1/2	—	0/1
Pool of lungs, liver, spleen, kidney.....	—	—	1/7
Pool of liver, spleen, kidney.....	—	3/5	—
Pool of liver, spleen.....	—	—	0/3
Lungs.....	0/2	0/4	—
Liver.....	0/2	—	0/6
Spleen.....	1/2	—	0/7
Kidney.....	0/2	—	—
Bladder.....	—	2/2	0/5
Adrenals.....	1/2	0/5	0/7
Lymph nodes			
Pooled cervical, submental.....	1/2	—	—
Cervical.....	—	2/6	1/10
Pooled axillary, inguinal.....	1/2	—	1/7
Axillary.....	—	1/2	1/3
Inguinal.....	—	2/4	0/2
Mesenteric.....	0/2	3/9	5/69
Mediastinal.....	—	—	0/1
Blood.....	0/2	1/1	(1/112)¶

\* Infected with murine-adapted strain; tissues tested in mice and cotton rats.

† Figures taken from the literature (1, 2); tests performed in monkeys.

‡ Figures taken from the literature (3-10); tests performed in monkeys.

|| Denominator = number of infected individuals tested; numerator = number of positive tests.

¶ The single positive test for virus in the blood is that reported from this laboratory previously (41).

tissues. Heart muscle was not tested in either the human or *cynomolgus* experiments of Sabin and Ward. The only other visceral organ yielding virus in the infant *rhesus* tests was the spleen. This was positive in the one *cyno-*

*molgus* spleen tested by Sabin and Ward and in a pool of lungs, liver, kidney, and spleen in one of their seven trials with human tissues. The fact that the spleen was positive in the baby *rhesus* in the absence of detectable virus in the blood suggests that the virus was actually in splenic tissue, and the positive result was not due to contamination by virus in the blood. However, the blood of infected *rhesus* monkeys (40) and in one instance that of a human being (41) have previously been found to contain virus.

The negative tests with the celiac plexus and the mesenteric lymph nodes are in line with some of the results in the human cases and the *cynomolgus*. The negative results with the pharyngeal wall and other tissues which might have been expected to be positive, may be attributed in part to the incomplete picture which results when only one animal has been studied.

#### SUMMARY

Although *rhesus* monkeys have been generally regarded as refractory to infection with poliomyelitis virus administered by the oral route, two of seven infant *rhesus* developed paralytic poliomyelitis when fed murine-adapted strains of virus. Preliminary intranasal treatment with zinc sulfate and negative serial sections of the olfactory bulbs of the positive animals ruled out the possibility that infection occurred by way of the olfactory pathway.

Studies on the distribution of virus in the tissues of the infected animals yielded positive results in one animal only. In this instance, virus was widely distributed throughout the body being isolated from spinal cord, buccal mucosa, duodenal wall, colon contents, superficial lymph nodes, spleen, heart, and adrenals.

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# PLASMA ESTERASE ACTIVITY IN PATIENTS WITH LIVER DISEASE AND THE NEPHROTIC SYNDROME

By HENRY G. KUNKEL, M.D., AND SYLVIA M. WARD

(From the Hospital of The Rockefeller Institute for Medical Research)

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An increasing amount of evidence has been accumulated (1-3) which indicates that the albumin of the plasma is formed in the liver. The fact that the degree of albumin depression correlates well with the severity of liver involvement in patients with various types of liver disease (4) is strong evidence in favor of such a concept. In view of the significance of the metabolic and oncotic effects of the albumin level of the plasma in determining the course of patients with various types of hypoproteinemia, it would appear to be of special importance to obtain information regarding albumin synthesis in these patients in an attempt to discover possible remediable defects. Direct studies of albumin turnover have yielded some information, but the picture in cirrhosis and nephrosis is often obscured by continued loss of albumin in ascitic fluid or urine. The therapeutic use of concentrated solutions of human albumin has made it even more difficult to determine the rate of production of albumin and to evaluate the changes brought about by such therapy. The use of radioactive and isotope techniques has not as yet been feasible. Two other plasma proteins, fibrinogen and prothrombin, also have been shown to be formed by the liver (5, 6). To this list it is now possible to add a fourth protein appearing in the plasma and synthesized by the liver, namely plasma esterase.

The possibility arose that, through a study in various hypoproteinemic states of the other proteins synthesized by the liver and their changes during therapy, information could be obtained regarding the general formation of proteins by the liver which might be applicable to the problem of albumin synthesis.

Of the group of liver proteins the plasma esterase lends itself most readily to accurate estimation. In addition, the regeneration of this enzyme can be studied in various pathological states, following its destruction by a parenteral injection of diisopropyl fluorophosphate (DFP). This material is an extremely powerful and specific inhibitor of the esterase group of enzymes. Several observers (7, 8) have demonstrated conclusively that these enzymes are irreversibly destroyed both *in vitro* and *in vivo* by DFP. Comroe, Todd, and Koelle (8), in connection with their use of this material in patients with myasthenia gravis, studied the regeneration of plasma esterase following its destruction by DFP. Wescoe (9) and Grob (10) have independently applied this technique to the study of patients with liver disease.

A decreased rate of formation of the enzyme was noted. These results correlated with the fact that the level of this enzyme in the plasma is markedly depressed in patients with involvement of the liver (11, 12). Other workers (13, 14) have demonstrated a high concentration of a non-specific esterase in the liver, similar to that found in plasma. The amount of this enzyme in the liver is depressed following experimental liver injury (13). These observations and others (15, 16) demonstrate quite conclusively that this enzyme is formed in the liver.

A close correlation between the plasma albumin level and the esterase activity in various pathological states was pointed out by Faber (11). Such a correlation did not exist in various proteinurias in which he found the plasma albumin to be depressed considerably more than the esterase (17). He offered as explanation that the amount of esterase lost in the urine in such patients, relative to albumin, was small and out of proportion to the plasma albumin-esterase ratio.

The present report represents a further study of the relationship between plasma albumin and esterase in patients with liver disease, with special emphasis on the effects of albumin therapy on the formation of these plasma components. In addition, the regeneration of this enzyme under various conditions in patients with the nephrotic syndrome was investigated and a comparison was attempted with the findings in the presence of a damaged liver. An indication of a defect in esterase synthesis in certain patients with the nephrotic syndrome was observed which was quite different from the defect found in patients with liver disease.

### *Materials and Methods*

Data reported in this communication were obtained from thirty patients with infectious hepatitis, twenty patients with cirrhosis of the liver, and ten patients with the nephrotic syndrome who were admitted to the Hospital or to the Out Patient Department of the Rockefeller Institute. The diagnosis of cirrhosis of the liver was clear from numerous liver function studies in addition to either the presence of ascites or direct visualization of biopsy sections of the liver. The ages of the patients with cirrhosis of the liver ranged from 12 to 60. The patients with the nephrotic syndrome all showed marked albuminuria, edema, ascites, and plasma albumin concentration below 2 gm. per cent. None of the patients showed a true lipoid nephrosis. They all showed evidence of glomerulonephritis at some time in their course. Their ages were all below 14. The normal controls included fourteen adults and six children below 12 years of age.

Plasma esterase was estimated by manometric measurement of the  $\text{CO}_2$  released from acetylcholine in bicarbonate buffer at pH 7.6. From 0.1 to 0.3 cc. plasma was diluted to 3 cc. with a buffer containing 0.03 M  $\text{NaHCO}_3$ , 0.12 M  $\text{NaCl}$ , and 0.04 M  $\text{MgCl}_2$ . This concentration of  $\text{NaHCO}_3$  in the presence of 5 per cent  $\text{CO}_2$  (in the gas phase) yields a pH of 7.6. The 3 cc. of diluted plasma was placed in the main compartment of Warburg flasks and acetylcholine chloride or bromide (0.5 cc. of 2.5 per cent solution in  $\text{H}_2\text{O}$ ) was placed in the side arm. The flasks were gassed with 5 per cent  $\text{CO}_2$  in  $\text{O}_2$  or  $\text{N}_2$  and allowed to equilibrate at  $38^\circ\text{C}$ . before mixing substrate

with plasma. After mixing, readings were taken every 5 minutes for 30 minutes and corrected for the change occurring in substrate and buffer alone. The results were expressed in terms of cubic millimeters of  $\text{CO}_2$  released per 1 cc. of plasma per minute. Tributyrin also was used as substrate. Inhibition experiments demonstrated that the enzyme in the plasma splitting this substrate was inactivated by DFP and prostigmine in a manner completely similar to the enzyme-splitting acetylcholine. In addition, the curve of regeneration of enzyme activity following DFP inactivation *in vivo* was the same with the two different substrates. It appeared that the same enzyme was measured in each case. The non-specific nature of plasma esterase was clear. Acetylcholine was used as substrate in the following experiments because it was hydrolyzed somewhat more readily and more accurate results could be obtained.

The enzyme was found to be extremely stable, and sera which had been stored for 2 years at  $0^\circ\text{C}$ . showed the same activity that had been found initially. Either serum or plasma could be used. No evidence of inhibitors of the enzyme in the plasma was ever found. Bile salts and pigments did not inhibit the enzyme in concentrations that exist in plasma. Patients with cirrhosis of the liver with or without jaundice showed equally great depression of esterase activity.

A preparation of DFP in peanut oil was used for the *in vivo* destruction of plasma esterase. 0.04 mg. per kilo of body weight was administered intramuscularly. Plasma esterase was determined the day following administration and at periodic intervals thereafter. The concentrated human serum albumin used therapeutically showed no esterase activity.

#### EXPERIMENTAL

The regeneration of plasma esterase following administration of DFP was studied in five individuals, two with the nephrotic syndrome, two with cirrhosis, and one normal (Fig. 1). It was apparent that the rate of regeneration of the enzyme differed markedly in these patients. The patients with cirrhosis showed a very slow formation of the enzyme, while those with the nephrotic syndrome synthesized the enzyme very rapidly. In each case the plasma esterase level approached the original concentration in approximately 30 days.

A close relationship appeared to exist between the rate of regeneration and the initial level of the enzyme in the plasma. It seemed as if the level of the enzyme in the plasma reflected its rate of formation. To test this hypothesis the absolute values used in constructing the widely divergent regeneration curves of Fig. 1 were expressed in terms of percentage of the initial concentration of the enzyme. When these figures were plotted against time (Fig. 2), very similar curves were obtained for each of the five patients. In other words, the percentage regeneration of the enzyme was the same; the wide difference in the absolute rate of regeneration in the patients was evident from the initial level of the enzyme in the plasma. The conclusion that the concentration of enzyme appearing in the plasma at any one time is a reflection

of the rate of formation of the enzyme seems justified. The major information regarding the synthesis of the enzyme can be obtained from its concentration

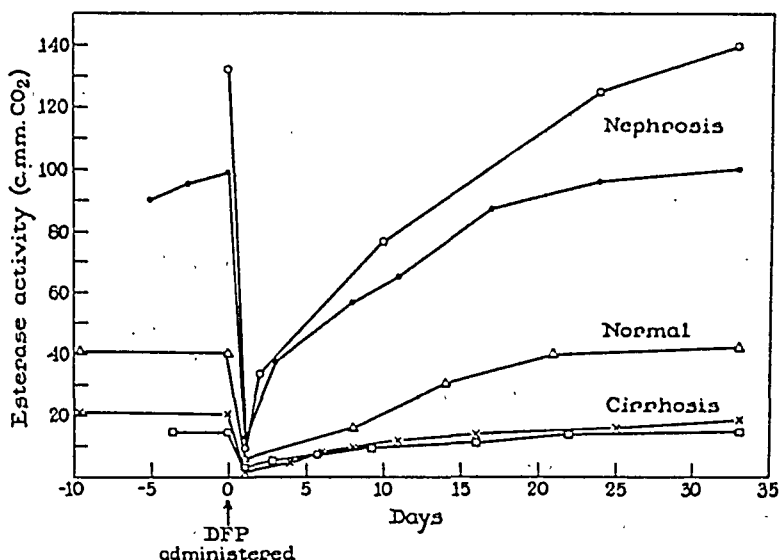


FIG. 1. Curves showing the regeneration of plasma esterase following the administration of DFP in five individuals, two with the nephrotic syndrome, two with cirrhosis of the liver, and one normal.

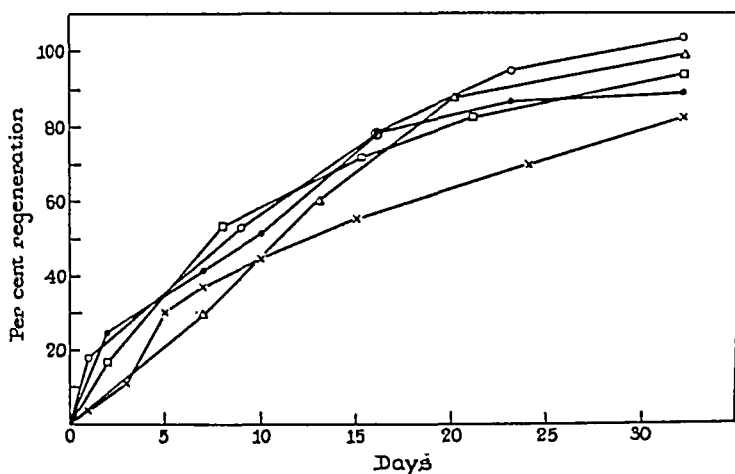


FIG. 2. Curves showing the plasma esterase regeneration following DFP with values expressed in terms of percentage of the initial esterase concentration in the same five individuals shown in Fig. 1.

in the plasma, and the difficult procedure of observing regeneration following DFP is unnecessary.

*Plasma Esterase and Albumin Concentrations in Patients with Acute In-*



*fectious Hepatitis*.—The relation of the esterase level to the albumin concentration of the plasma was studied in thirty patients with acute infectious hepatitis. This disease offered the ideal opportunity for investigating the aberrations following acute liver damage. Forty-four per cent of this group showed an albumin level below 4 gm. per cent which returned to normal late in convalescence. All of the patients showing abnormalities in the amount of plasma albumin also showed lowered esterase values (Table I). The degree of lowering was somewhat difficult to determine because the esterase values tended to rise above normal during convalescence, and in order to determine the normal for each individual it was necessary to obtain values several months after the illness had terminated. The wide variation of esterase concentration in healthy individuals (40 to 90 c.mm. CO<sub>2</sub>) made it essential to obtain the normal for each person. A depression of less than 10 c.mm. CO<sub>2</sub> was not considered significant. Seventeen patients or 57 per cent of the thirty cases

TABLE I

*Comparison of Plasma Esterase Depression in Infectious Hepatitis Patients with and without Abnormalities in Plasma Albumin*

	No. of patients	Per cent	Per cent showing esterase depression >10	Average maximal esterase depression c.mm. CO <sub>2</sub>
Plasma albumin <4 gm. per cent.	13	43	100	28
Plasma albumin >4 gm. per cent (normal).....	17	57	46	18

showed no change in albumin concentration during the course of the disease. The esterase depression was considerably less in this group as shown in Table I.

These observations demonstrate that the plasma esterase is more readily depressed in the presence of liver damage than the albumin level but that some relationship does exist. The fact that the patients with a depression of plasma albumin were the most severely ill generally should be considered in evaluating this relationship. Of greater significance was the finding that the depression of albumin and esterase occurred at approximately the same period of the disease. Both showed a delayed fall at a time when the symptoms of the illness were subsiding but prior to the rise in the globulin level (18). Table II illustrates this relation in two typical cases.

*Plasma Esterase Activity in Patients with Cirrhosis of the Liver and the Changes Occurring with Therapy*.—In patients with cirrhosis of the liver the esterase depression was much more marked than in those with infectious hepatitis and the concentration was always below the normal range of 40 to 90 c.mm. CO<sub>2</sub>. Table III illustrates the results in eighteen patients with cirrhosis of the liver with edema and ascites. It is apparent that the patients with the lowest

albumin levels also had the most marked depression of the esterase concentration. Serial determinations of plasma esterase over long periods of time in these patients demonstrated that the esterase levels remained very constant. The patients often improved markedly on various types of therapy without any significant change in esterase activity. It was only in those few patients who regained the ability to synthesize normal amounts of plasma albumin

TABLE II

*Comparison of Serial Determinations of Plasma Esterase and Albumin in Two Patients with Infectious Hepatitis*

Day of disease	Plasma esterase	Plasma albumin	Day of disease	Plasma esterase	Plasma albumin
	<i>c.mm. CO<sub>2</sub></i>	<i>gm. per cent</i>		<i>c.mm. CO<sub>2</sub></i>	<i>gm. per cent</i>
10 days before	68	4.1	2	60	4.0
2	70	4.3	3	63	4.2
5	52	3.6	6	41	3.5
7	50	3.7	10	32	3.5
12	68	4.0	18	54	4.0
19	76	4.2	25	74	4.2
27	75	4.1	90	64	4.1
34	80	4.1			
56	72	4.3			

TABLE III

*Comparative Results of Plasma Esterase Determinations in Patients with Cirrhosis of the Liver with Different Concentrations of Serum Albumin (All Had Ascites and Edema)*

	No. of patients	Albumin level	Average esterase level
		<i>gm. per cent</i>	<i>c.mm. CO<sub>2</sub></i>
Group A.....	13	2-3	22
Group B.....	5	1-2	13

that a rise in esterase occurred. Fig. 3 illustrates the results of serial determinations of serum albumin and esterase over a period of 18 months in a patient with cirrhosis of the liver. The patient received large amounts of albumin intravenously, which brought about marked clinical improvement with loss of ascites and edema after approximately 4 months of therapy. However, he was unable to synthesize sufficient albumin to preserve permanently the normal serum albumin level provided by the injections. The plasma esterase concentration remained constant throughout this period. After approximately 12 months of continued general improvement, during which time he put on considerable body weight, he finally regained the capacity to maintain a

normal plasma albumin level. At precisely the same time he demonstrated the capacity to synthesize larger amounts of plasma esterase. Following prolonged supportive therapy, the function of the liver finally improved sufficiently to enable it to synthesize significant amounts of these two proteins. Two other patients with cirrhosis of the liver who were treated with intravenous liver extract for prolonged periods also regained their ability to preserve a normal plasma albumin level as well as a normal esterase level. Ten patients with cirrhosis of the liver who have not regained the ability to form albumin in a normal manner have shown no rise in plasma esterase although considerable clinical improvement was evident in the majority. In every case the serum esterase remained abnormal as long as a defect in albumin synthesis persisted.

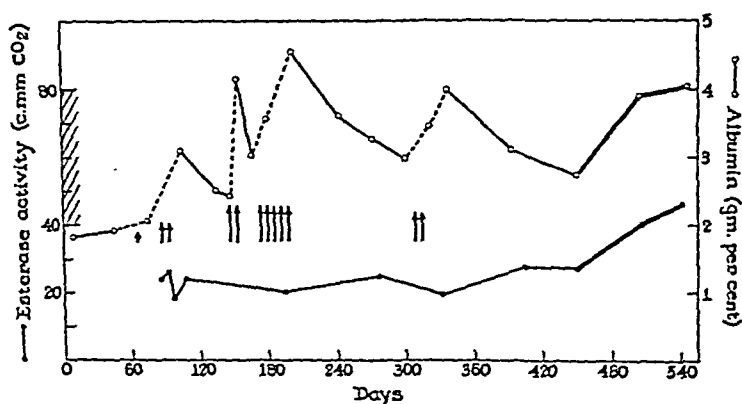


FIG. 3. Curves showing the influence of long continued intravenous administrations of concentrated human serum albumin on the plasma albumin and esterase levels in a patient with cirrhosis of the liver.

*Plasma Esterase Activity in Patients with the Nephrotic Syndrome.*—In direct contrast to the findings in patients with liver disease, the esterase levels in patients with the nephrotic syndrome usually are higher than normal. Table IV shows the average results from ten patients. For purposes of comparison the results from normal individuals and patients with cirrhosis of the liver are added. Fig. 1 illustrates the rapid regeneration of this enzyme in two patients with the nephrotic syndrome.

In order to obtain information as to the reasons for the high values for plasma esterase in the nephrotic syndrome, serial determinations were carried out over prolonged periods of time in such patients and variations during different phases of the disease were studied. Fig. 4 illustrates the results of serial determinations of plasma esterase over a period of 3 months in two children with severe nephrosis.

Both patients had marked edema and ascites with severe albuminuria and a serum albumin below 1 gm. per cent. Neither patient received any specific therapy. In each case the esterase values persisted above normal as long as the patients were followed. Patient 1 showed no evidence of improvement in his general condition

TABLE IV

*Comparative Amounts of Plasma Esterase in Patients with the Nephrotic Syndrome, Cirrhosis of the Liver, and in Normal Controls*

	No. of cases	Highest value	Lowest value	Average value
Nephrosis.....	10	180	45	110
Cirrhosis.....	18	30	6	19
Normal controls.....	20	91	42	65

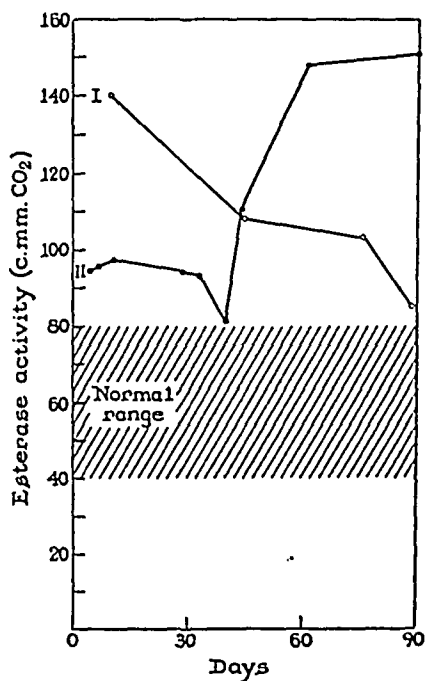


FIG. 4. Curves showing the results of serial determinations of plasma esterase in two children with the nephrotic syndrome. Patient 1 died, while Patient 2 developed a spontaneous remission.

and following a series of secondary infections finally died. His esterase values showed a gradual decline reaching a minimum at the time of death. Patient 2 also showed little change at first but suddenly, after approximately 3 months in the hospital, he developed a diuresis with loss of 10 kilos of body weight and showed a gradual rise in his plasma albumin level. This improvement was accompanied by a rise in his plasma esterase which closely paralleled the rise in plasma albumin.

Similar increases of the enzyme also were observed in two other patients with nephrosis who had a spontaneous improvement in their general condition. The rise in esterase was associated with an increase in plasma albumin despite persistent albuminuria.

*Effect of Albumin Therapy on the Esterase Level of the Plasma of Patients with the Nephrotic Syndrome.*—In view of the rise of plasma esterase following a spontaneous remission in patients with nephrosis, it seemed advisable to ob-

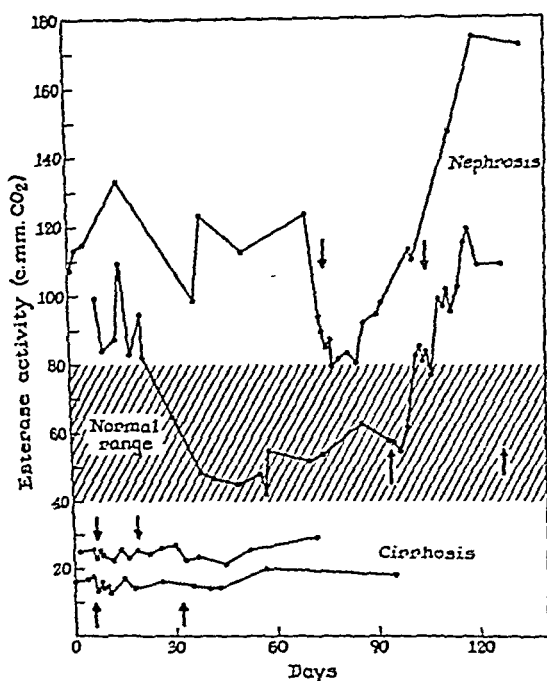


FIG. 5. Curves showing the effect of intravenous albumin administration on the plasma esterase level in two patients with the nephrotic syndrome and two patients with cirrhosis of the liver. The arrows indicate the beginning and end of therapy in each case.

serve the changes in the enzyme level produced by various types of therapy. Determinations were carried out serially in two patients with the nephrotic syndrome who had very low plasma albumin levels and who were treated intravenously for approximately 30 days with an average of twenty-four 25 gm. units of concentrated human albumin (Fig. 5). For purposes of comparison, the results in two patients with cirrhosis of the liver who were similarly treated are charted. The patients with the nephrotic syndrome had a striking rise in the concentration of esterase which began approximately 10 days after the onset of therapy. This was of special interest because the patients im-

proved considerably symptomatically with little other laboratory evidence of improvement. In contrast to the patients with the nephrotic syndrome, the patients with cirrhosis of the liver showed very little variation in the plasma esterase level despite clinical improvement.

#### DISCUSSION

The chief value of the technique of observing the regeneration of plasma esterase following destruction by DFP was to demonstrate that the level of the enzyme in the plasma reflects its rate of formation. Added significance may now be given to the concentration of the enzyme in the plasma in various disease states. The rapid regeneration of plasma esterase in patients with the nephrotic syndrome as compared with the very slow regeneration in patients with cirrhosis of the liver could be estimated as well by the determinations of the level of the enzyme in the plasma as by the time-consuming technique using DFP.

The low levels of plasma esterase in patients with severe liver disease appeared to reflect the inability of the liver to form normal amounts of this enzyme. The liver's ability to form another protein, albumin, was similarly affected. The delayed fall in these two proteins following acute liver damage also suggested a formation defect. The degree of depression was proportional to the severity of the liver damage. In patients with cirrhosis of the liver long sustained therapy is necessary before the liver regains the ability to synthesize normal amounts of albumin and esterase. The fact that the time necessary for this to occur was approximately the same for the two proteins is perhaps the best evidence of a similar formation. In these patients there was little doubt about the site of the defect in protein synthesis. The damaged liver is unable to perform this function adequately regardless of the availability of the essential materials necessary for protein production.

Numerous investigators have postulated a possible defect in protein synthesis by the liver in patients with the nephrotic syndrome (19-22). The frequent observation of low plasma albumin levels in certain patients losing an amount of albumin that the normal person could readily replace has led to the belief that the primary defect may lie in the formation of albumin. The application of the usual liver function tests, however, has not revealed any abnormality of the liver in these patients (23). Moreover, since the formation of plasma esterase is dependent on the condition of the liver, the high values found in patients with the nephrotic syndrome suggest that the liver is performing this function in a hypernormal fashion. Although this evidence argues against a primary hepatic defect in protein formation in nephrosis, it is of interest that albumin replacement therapy leads to rises in esterase values to even higher levels, as is shown also during spontaneous remissions.

It seems possible that the hypernormal levels of plasma esterase in nephrosis

reflect a general response on the part of the liver to regenerate protein more rapidly as a result of the loss of albumin in the urine. Further evidence for this hypothesis can be found in the high concentrations of fibrinogen found in the plasma of these patients (24). As with plasma esterase, fibrinogen is synthesized by the liver and is depressed in patients with severe liver involvement. Electrophoretic studies have shown that certain of the alpha and beta globulins may rise in patients with nephrosis (25). It is not known whether all of these proteins are synthesized in the liver. Immunological studies have also revealed high concentrations of unusual proteins precipitating with the albumin fraction of the serum of these patients. On the other hand, the gamma globulins which are generally considered to be formed independently of the liver are actually depressed (25); in liver disease they are increased (26). It would appear, therefore, that in patients with nephrosis the liver is functioning well and is stimulated by the albumin loss to form unusual amounts of the proteins which it synthesizes. The question whether some of the stimulus comes as a response to furnish proteins for the maintenance of the oncotic pressure of the blood in the absence of albumin cannot be answered from the data at hand.

The rapid rise in plasma esterase concentration following spontaneous remissions of the nephrotic syndrome or following therapy with albumin, when contrasted with the difficult alterability of the enzyme concentration in patients with cirrhosis, demonstrates quite strikingly the extreme difference between these two disease states. The fact that a rise in esterase concentration does occur in certain patients with the nephrotic syndrome demonstrates that a defect in esterase synthesis may be present. This defect would appear to be very different from that existing in patients with cirrhosis of the liver. The rapidity of the esterase change following albumin therapy in patients with the nephrotic syndrome strongly suggests that the albumin supplies some factor which in the presence of an adequately functioning liver brings about increased synthesis of the enzyme. The essential factor may very well be albumin itself.

The defect in esterase formation may be similar to that which is thought to exist in the synthesis of albumin in the severely ill nephrotic patient. Since the synthesis of albumin is obscured by the constant loss of albumin in the urine, study of the plasma esterase system yields information concerning a similar protein, not lost in the urine to a similar extent, but the synthesis of which is altered by the conditions underlying the nephrotic syndrome. It may be possible by this means to define the exact defect in protein formation that exists in these patients.

The studies here reported appear to indicate that the liver is not primarily at fault but that certain essential materials which the liver needs for the synthesis of proteins in adequate amounts are lacking in certain patients with the

nephrotic syndrome. If these are supplied, the liver can readily form the necessary protein. Evidence was presented that albumin administration aids in the synthesis of esterase by the liver. It may well be that a certain amount of albumin must also be present in the blood and tissues for proper synthesis of albumin by the liver.

#### SUMMARY

By a study of plasma esterase in various hypoproteinemic states information was gained concerning the synthesis of a protein by the liver, which may be applicable to the problem of albumin synthesis. Patients with infectious hepatitis and cirrhosis showed defective formation of plasma esterase that paralleled the defect in albumin formation. The defect could only be altered in patients with cirrhosis by very prolonged therapy indicating that liver function itself had to improve before the proteins could be formed in a normal manner. Patients with the nephrotic syndrome showed a normal or hyper-normal formation of plasma esterase. Following spontaneous remissions or the administration of albumin the esterase level showed a marked rise which was in direct contrast to the difficult alterability of the enzyme level in patients with severe liver involvement. It is suggested that the defect in protein synthesis by patients with the nephrotic syndrome may be due to the lack of certain essential materials, one of which may be albumin itself, rather than to any abnormality in the liver.

It is a pleasure to acknowledge the aid of Dr. F. P. Chinard, Dr. G. C. Cotzias, Dr. H. A. Eder, and Dr. O. J. Malm, and the helpful suggestions of Dr. D. D. Van Slyke.

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# THE PATHOGENESIS OF THE RENAL INJURY PRODUCED IN THE DOG BY HEMOGLOBIN OR METHEMOGLOBIN\*

By H. E. HARRISON, M.D., H. BUNTING, M.D., N. K. ORDWAY, M.D., AND  
W. S. ALBRINK, M.D.

*(From the Department of Pathology, Yale University School of Medicine, New  
Haven)*

PLATES 32 TO 34

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Serious impairment of renal function may be found associated with the hemoglobinuria resulting from intravascular hemolysis or the injection of solutions of hemoglobin (1, 2). Similar disturbances of kidney function are observed following the crush injury of skeletal muscle with liberation of myoglobin from the injured muscle and excretion of metmyoglobin in the urine (3). Three general hypotheses have been suggested to explain this type of renal injury: obstruction of the renal tubules by the precipitation of derivatives of hemoglobin or myoglobin in their lumina (1), injury of renal tubule cells by toxic concentrations of these heme pigments (4-6), and diminished renal blood-flow due to vasoconstriction of renal blood vessels (7, 8). Various modifications and elaborations of all of these hypotheses have been proposed and the possibility that all three mechanisms may be involved in varying degrees has been appreciated.

During the course of experiments designed to study methods of treatment of arsine poisoning, the pathogenesis of the renal dysfunction resulting from the intravascular hemolysis produced by arsine was investigated. Experiments were also carried out on dogs in which renal injury was produced by the intravenous injection of solutions of dog hemoglobin or methemoglobin.

Dogs were placed in a static<sup>1</sup> gassing chamber in which arsine was generated by the reaction of magnesium arsenide with water. The concentration of arsine in the chamber air was controlled by the amount of magnesium arsenide introduced into the reaction vessel. A concentration of 1 mg. of arsine per liter of air was most commonly used and the mortality rate of dogs exposed to this concentration for 15 minutes was 79 per cent.

Solutions of dog hemoglobin were prepared by the method of Hamilton and Van Slyke (9). Washed dog red blood cells were hemolyzed by the addition of distilled water, and the stroma proteins were precipitated by acidification of the solution to pH 5.8 with 0.1 N HCl. Following centrifugalization and filtration of the supernatant through filter paper, the clear solution was

\* The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Yale University, July 1, 1944, to December 31, 1945.

<sup>1</sup> In the static type of gassing chamber there is no exchange of air during the period of exposure of the animal to the toxic vapor.

brought back to pH 7.0 by the addition of 0.1 N NaOH and sterilized by filtration through a Seitz filter. Solutions of methemoglobin were prepared by the addition of 4 moles of  $\text{NaNO}_2$  per mole of hemoglobin. The concentrations of hemoglobin and methemoglobin in the solutions and in the plasma of the experimental animals were determined by the method of Evelyn and Malloy (10).

The effects of intravascular hemolysis or of administration of solutions of hemoglobin and methemoglobin upon kidney function were measured by serial determinations of the concentration of urea nitrogen and creatinine in the plasma, and by determinations of the creatinine clearances. Urea nitrogen was determined by the manometric method of Van Slyke and Kugel (11) and creatinine by the Folin and Wu method (12) adapted for the photoelectric colorimeter.

An adaptation of Gersh's histochemical technique (13) for the study of kidney function was utilized in an attempt to determine whether the renal tubules were functionally obstructed. The details of the method as used are described elsewhere (14). Following the intravenous injection of solutions of sodium ferrocyanide, the distribution of ferrocyanide can be visualized in sections of kidney frozen and dehydrated *in vacuo*. In the dog, ferrocyanide is filtered through the glomerular membrane and is not reabsorbed by the renal tubule cells (15). Thus, in the kidney of the normal dog, ferrocyanide can be demonstrated histochemically in the glomerular space and lumen of the tubule while the tubule cells do not contain ferrocyanide ion. If the tubule cells are injured by the administration of known cytotoxic agents such as mercuric chloride, the damaged cells can be seen to contain ferrocyanide. Mechanical obstruction to the flow of urine through the tubules is indicated by failure to demonstrate ferrocyanide ion in the distal portions of the tubular lumina.

The kidney sections were also examined following fixation and staining, and the number of pigment casts per unit area in the sections of kidney stained with hematoxylin and eosin following fixation in Zenker formol solution was counted. Benzidine and iron-alum-hematoxylin stains were used to demonstrate the presence of hemoglobin or methemoglobin (16, 17).

Studies were made of the nature of the hemoglobin derivative comprising the pigment of the casts. The casts were teased out of frozen sections of unfixed kidney tissue, dissolved in buffer solutions, and the absorption spectrum of the pigment examined by means of a Beckman spectrophotometer. The solubilities of the casts in buffer solutions of varying pH were also determined in a qualitative fashion.

Direct measurements of total renal blood flow were made in anesthetized dogs before and after the intravenous injection of solutions of dog methemoglobin. The left kidney of a dog anesthetized with sodium pentobarbital was brought out through a flank incision without tension on the pedicle and fixed with sutures beneath the skin. Following the intravenous injection of heparin, a T-tube cannula was inserted into the renal vein and tied in place, permitting unrestricted flow of blood through the cannula into the inferior vena cava. To determine the rate of blood flow through the kidney, the segment of renal vein between the cannula and the vena cava was occluded with simultaneous opening of the side arm of the T-tube. The blood flowing out of the side arm for a period of time (measured by stopwatch) was collected and its volume measured. The side arm was then closed, and the constriction of the renal vein released, restoring the flow into the vena cava. Repeated determinations were made and the blood removed was returned to the circulation whenever 30 or 40 cc. had been collected.

### *Methods*

#### *Studies of Renal Function and Renal Blood Flow*

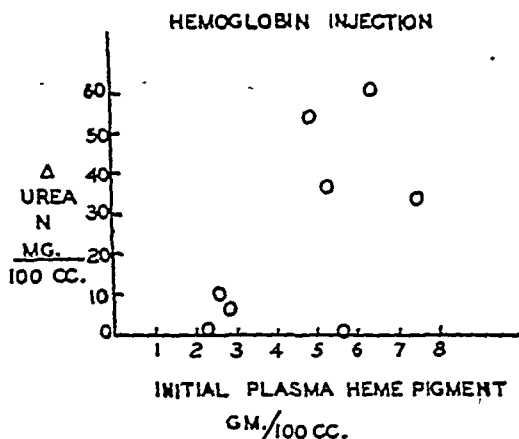
In the dog intravascular hemolysis produced by poisoning with arsine may cause impairment of kidney function as indicated by a progressive rise of the

concentration of urea nitrogen in the plasma. The dog was found, however, to be extremely resistant to the injurious effects of hemoglobinemia and hemoglobinuria, and renal injury could be demonstrated only in animals with extreme hemolysis. The findings in two dogs, which survived over 48 hours following exposure to arsine and developed evidences of renal injury are given in Table

TABLE I  
*Course of Events in Dogs Exposed to Arsine*

Dog No.	Packed R. B. C. volume* 24 hrs. post gassing	Plasma hemoglobin 24 hrs. post gassing	Blood urea N 48 hrs. post gassing
	<i>per cent</i>	<i>gm./100 cc.</i>	<i>mg./100 cc.</i>
14-48	10	3.9	122
14-47	35	6.7	161
15-34	51	2.0	21
15-40	24	4.0	24

\* Expressed as per cent of the pregassing volume.

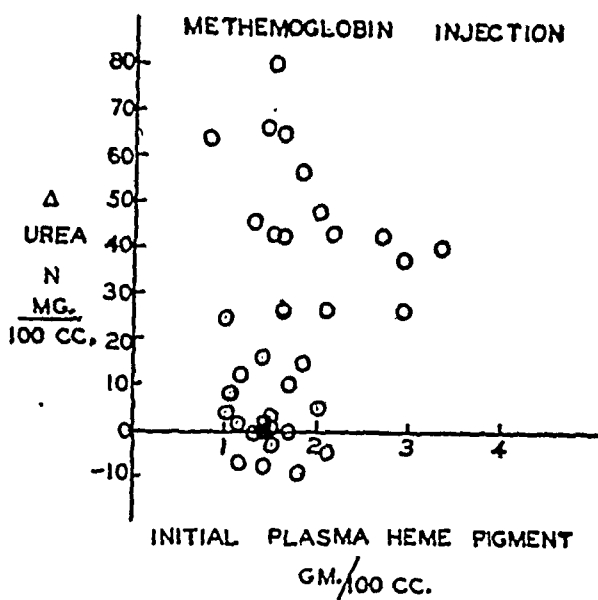


TEXT-FIG. 1. Relation of initial concentration of plasma heme pigment to rise in blood urea N during 24 hours following injection of hemoglobin solution.

I, in comparison with those of two dogs which survived without evidences of renal damage. The degree of hemolysis of the red cells is indicated by the decrease in the packed red cell volume and by the concentration of hemoglobin in solution in the plasma.

The experiments in which solutions of dog hemoglobin were injected into normal dogs also indicated that disturbance of kidney function could be produced only by injections of large amounts of hemoglobin in concentrated solution. In Text-fig. 1, the initial concentration of hemoglobin in the plasma obtained 3 to 5 minutes following intravenous injection of a solution of hemo-

globin is plotted against the change in concentration of urea nitrogen in the plasma during the first 24 hours following a single injection of hemoglobin. A progressive increase in the plasma urea nitrogen was found in dogs which had received sufficient hemoglobin to raise the plasma hemoglobin concentration to approximately 5 gm. or more per 100 cc. The apparent lack of injurious effect of the lower concentrations of hemoglobin is emphasized by the fact that four of the dogs in which little or no rise in plasma urea nitrogen was found had



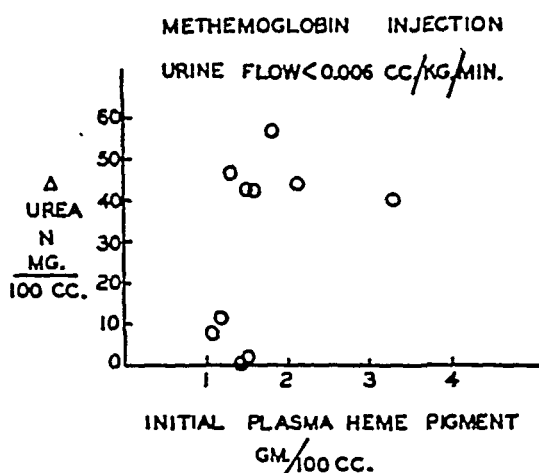
TEXT-FIG. 2. Relation of initial concentration of plasma heme pigment to rise in blood urea N during 24 hours following injection of methemoglobin solution.

been made oliguric by deprivation of water and their urine flow had been reduced to 0.0057 cc. per kilogram per minute or less prior to the injection of hemoglobin.

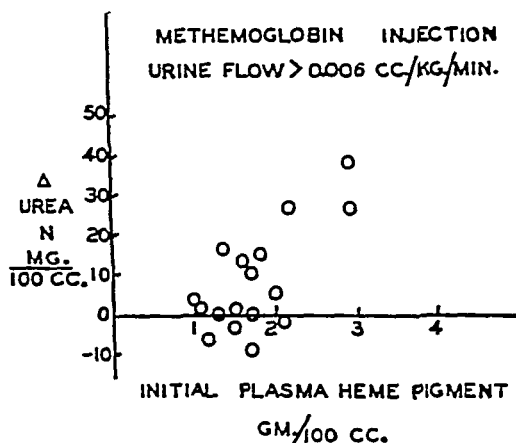
Bing (18) has reported that a severe degree of renal injury could be produced by the injection of methemoglobin solutions into dogs made acidotic by the administration of ammonium chloride. Comparable amounts of hemoglobin injected into acidotic dogs had little effect upon kidney function and the injection of methemoglobin into normal dogs also failed to reduce kidney function. We determined the effects of injection of methemoglobin into normal dogs, dogs made oliguric by deprivation of water, and dogs made acidotic by intragastric administration of 0.1 N hydrochloric acid for several days. The experiments in all of the non-acidotic dogs are summarized in Text-fig. 2 in which the initial concentration of plasma heme pigment (methemoglobin plus hemoglobin)<sup>2</sup> is plotted against the change in concentration of urea nitrogen

<sup>2</sup> The solutions of methemoglobin employed in many instances contained some unoxidized hemoglobin.

in the plasma during the 24 hours following the injection of the methemoglobin solution. The apparent lack of correlation between concentration of heme

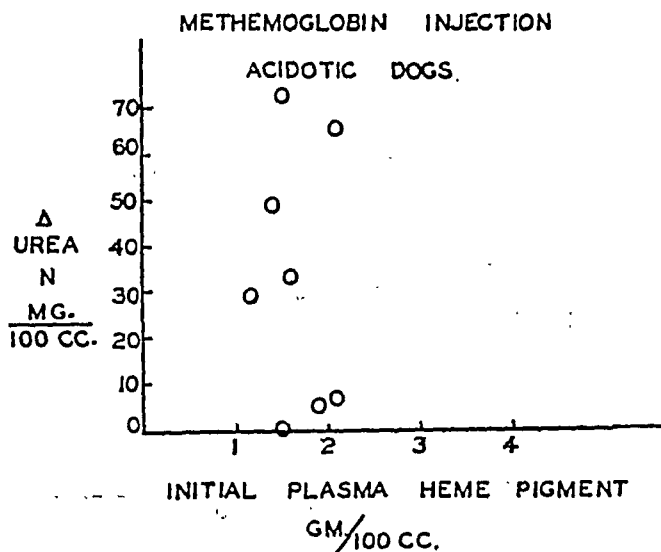


TEXT-FIG. 3. Relation of initial concentration of plasma heme pigment to rise in blood urea N during 24 hours following injection of methemoglobin solution into oliguric dogs with urine flow of less than 0.006 cc. per kilo per minute at time of injection.



TEXT-FIG. 4. Relation of initial concentration of plasma heme pigment to rise in blood urea N during 24 hours following injection of methemoglobin solution into dogs with urine flow greater than 0.006 cc. per kilo per minute.

pigment in the plasma and the elevation of the blood urea nitrogen is explained when the results are separated into 2 groups: dogs with urine flow below 0.006 cc. per kilo per minute at the time of injection and those with urine output greater than this value. In Text-fig. 3 the data are given for the dogs deprived



TEXT-FIG. 5. Relation of initial concentration of plasma heme pigment to rise in blood urea N during 24 hours following injection of methemoglobin solution into acidotic dogs.

TABLE II

*Clearances of Endogenous Creatinine in Oliguric Dogs before and after Injection of Hemoglobin*

Elapsed time	Urine flow	Plasma creatinine	Creatinine clearance	Plasma hemoglobin
min.	cc./min.	mg./100 cc.	cc./min.	gm./100 cc.
Dog 16-28, weight 9.3 kg.				
0-28	0.033	0.98	15.1	—
28-33	Intravenous injection of 37 gm. hemoglobin			—
33	—	—	—	5.3
33-58	Trace	—	—	—
58-119	0.20	1.35	4.6	—
119-188	0.53	1.45	6.8	—
1274	—	—	—	1.35
1274-1340	0.11	2.04	7.4	—
Dog 16-57, weight 5.9 kg.				
0-90	0.028	0.72	17.0	—
96-100	Intravenous injection of 24 gm. hemoglobin			—
100	—	—	—	4.9
100-311	Trace	—	Trace	—
311	—	1.58	—	—
1346-1418	0.056	3.13	0.29	—

of water with reduction of urine flow to 0.006 cc. per kilo per minute or less at the time of injection of methemoglobin and in Text-fig. 4 are plotted the results



for dogs with urine flows in excess of this value. In the oliguric dogs severe impairment of renal function is seen following the injection of methemoglobin

TABLE III

*Clearances of Endogenous Creatinine in Oliguric Dogs before and after Injection of Methemoglobin*

Elapsed time	Urine flow	Plasma creatinine	Creatinine clearance	Plasma heme pigment
min.	cc./min.	mg./100 cc.	cc./min.	gm./100 cc.
Dog 16-45, weight 10.4 kg.				
0-96	0.057	0.70	25.9	—
100-104	Intravenous injection of 9.9 gm. methemoglobin			—
106	—	—	—	1.65
106-145	Trace	—	—	—
145-232	0.037	1.07	0.5	—
232-310	0.088	1.25	1.4	—
310-380	0.049	1.37	1.5	—
Dog 16-29, weight 8.2 kg.				
0-81	0.058	1.31	19.7	—
81-90	0.037	1.31	15.5	—
	Intravenous injection of 8.2 gm. methemoglobin			—
99	—	—	—	1.82
99-435	Trace	—	Trace	—
435	—	2.59	—	—

TABLE IV

*Clearances of Endogenous Creatinine in Acidotic Dog before and after Injection of Methemoglobin*

Elapsed time	Plasma CO <sub>2</sub>	Urine flow	Urine pH	Plasma creatinine	Creatinine clearance	Plasma heme pigment
min.	mm./liter	cc./min.		mg./100 cc.	cc./min.	gm./100 cc.
Dog 14-19, weight 17.2 kg.						
0-89	10.0	0.37	5.3	0.74	36.8	—
	Intravenous injection of 13.9 gm. methemoglobin					—
104						1.37
104-140		1.19	6.0	0.84	21.1	
140-192		0.54	6.0	0.94	10.8	
192-299		0.52	—	1.08	9.7	
299-403		0.48	—	1.22	8.3	

in amounts which result in an initial plasma pigment concentration of approximately 1 gm. per 100 cc. or more, whereas in the dogs with the greater urine output much greater concentrations of plasma methemoglobin are found without severe renal injury.

The results of the experiments in the acidotic dogs are similarly plotted in Text-fig. 5. At first glance it appears that an acidosis of moderate severity ( $\text{CO}_2$  content of serum 10 to 15 mm per liter) did not intensify the injurious effect of methemoglobin upon kidney function. The rate of urine flow in these dogs at the time of injection of methemoglobin was, however, much greater than in the non-acidotic animals. Although acidosis probably did have some effect in increasing the kidney damage resulting from injection of methemoglobin, in these experiments the rate of urine flow was the more important factor.

Creatinine clearances were determined in these dogs before and after the injection of solutions of hemoglobin or methemoglobin. Because of the low rates of urine flow endogenous creatinine clearances were determined, and the individual collection periods were usually 30 minutes or longer. The urine was collected by an indwelling catheter, and the bladder was carefully washed at the end of each period and the washings added to the urine sample. Typical experiments are tabulated in Tables II, III, and IV. Immediately following the injection of hemoglobin in a dosage of about 4 gm. per kilo or methemoglobin in a dosage of approximately 1 gm. per kilo into the oliguric dog, urine flow may abruptly cease. After a variable period of time measurable amounts of urine can be collected, but as the results given in Tables II and III indicate, the creatinine clearances are reduced to extremely low levels. In the experiment on the acidotic dog with normal urine flow (Table IV) no period of anuria was observed following the injection of methemoglobin but the creatinine clearance dropped rapidly and progressively. The urine pH rose immediately following the injection of methemoglobin and this phenomenon has been seen in all of the acidotic dogs. In several of the experiments the urine pH rose from 5.2 or 5.3 to approximately 7.0 at the onset of hemoglobinuria. A similar rise in urine pH with onset of hemoglobinuria was found in dogs exposed to arsine.

Because of the abrupt drop in the creatinine clearance seen in many of these experiments the possibility of marked reduction of renal blood flow following the injection of methemoglobin or hemoglobin was considered. Direct measurements of renal blood flow by the technique described above were made in two dogs injected with methemoglobin. The protocols of these experiments are given in Table V. No evidence of reduction of blood flow through the kidneys was found except after prolonged anesthesia and manipulation. An increase of renal blood flow was seen immediately following the injection of methemoglobin solutions, which was probably due to the increase of plasma volume resulting from the injection of a 5 per cent solution of methemoglobin. One of the animals, CK 8, was essentially anuric at a time when total renal blood flow was normal.

TABLE V

*The Effect of Intravenous Injection of Methemoglobin on Renal Blood Flow*

Elapsed time	Urine output	Renal blood flow
min.	cc./min.	cc./min.
Dog CK 8, weight 6.1 kg.		
0	Anesthetized with pentobarbital—renal vein cannulated	
9	0.041	
23		51.0
35		52.8
41		47.6
47		52.1
52		52.0
57		
57-60	Intravenous infusion of 114 cc. 5.3 per cent methemoglobin solution	
64	Too little to measure*	62.8
66		62.1
74		52.3
79		49.0
85		
103	Too little to measure*	58.1
115		55.2
157		27.6
159		24.0
163	Experiment terminated	28.8
165		
Dog CK 9, weight 6 kg.		
0	Anesthetized with pentobarbital—renal vein cannulated	
10	0.049	
43		96
44		140
45		108
50		97
66		81.6
86		125
93		96
96		99.4
99		122
104		
117-120	Intravenous infusion of 140 cc. of 4.2 per cent methemoglobin solution	
121		173

TABLE V—*Continued*

Elapsed time	Renal blood flow	Urine output
min.	cc./min.	cc./min.
Dog CK 9, weight 6 kg.— <i>Concluded</i>		
122	0.17†	162
127		138
142		151
171		84.5
205		81.3
256	0.09†	62.1
262		84
265		Experiment terminated

\* Bladder washings contained methemoglobin.

† Methemoglobin in urine.

### *Histological Findings*

The kidneys of dogs examined 2 hours or more after the onset of hemoglobinuria due to exposure to arsine or following the injection of solutions of hemoglobin or methemoglobin were found to contain an eosinophilic granular precipitate within the glomerular spaces and the lumina of the tubules. This material stained greenish brown with benzidine or alum-hematoxylin as did the hemoglobin within the red blood cells. By 11 to 24 hours after exposure to arsine or 1½ to 2 hours after the intravenous injection of hemoglobin or methemoglobin, masses of granular yellow-brown refractile material and well formed casts of similar appearance were found within the loops of Henle, the distal convoluted tubules, and collecting tubules (Fig. 1). In the kidneys of animals which showed marked hemoglobinuria, the epithelium of the proximal convoluted tubules contained hyaline eosinophilic droplets which stained greenish brown with benzidine or alum-hematoxylin. This material presumably represented hemoglobin or a derivative which had been absorbed by the epithelial cells from the glomerular filtrate (Fig. 2). Only an occasional necrotic cell was seen in tubules plugged with pigment casts (Fig. 3). Some of the loops of Henle contained eosinophilic casts that did not stain as did hemoglobin but resembled non-hemoglobin protein. A few dilated tubules lined by flattened epithelium could be found among the large number of cast-filled tubules.

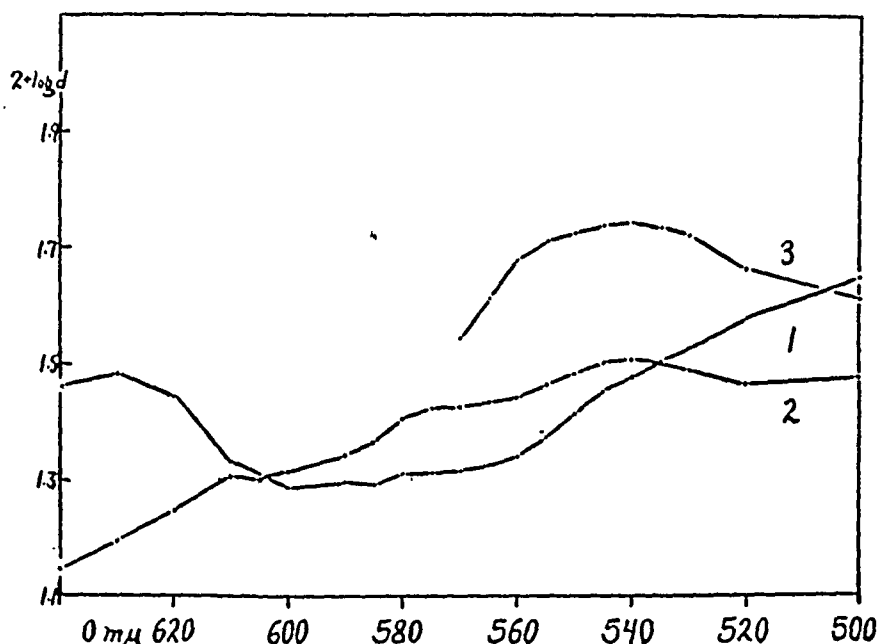
By 36 to 48 hours after the onset of hemoglobinuria, coagulative necrosis of some of the proximal convoluted tubule cells was found in the kidneys of animals in which very extensive intravascular hemolysis had occurred or in those that had received large amounts of methemoglobin intravenously. At times

only a few cells of a tubule were necrotic while the remainder contained in their cytoplasm the eosinophilic droplets described above. Groups of dilated tubules lined by flattened cells were usually present. Regeneration of tubular epithelium was first seen at 48 hours, appearing as a flattened layer of cells surrounding a granular necrotic mass. Intracellular granules containing free iron as indicated by the Prussian blue reaction were seen rarely at this stage but became conspicuous later. The cast material was found to give a positive Prussian blue reaction; occasionally this was limited to the periphery of the cast.

Three or 4 days after the onset of hemoglobinuria, the number of casts was reduced. Radial zones of tubules containing casts alternating with sectors free of casts were often seen (Fig. 4). The tubules of the latter zones frequently were dilated. In other instances few casts were found although there were alternating sectors of dilated and collapsed tubules. The collapsed tubules were lined by tall cuboidal cells containing hemosiderin. This picture was essentially that found in animals examined at later periods except that casts became progressively fewer (Fig. 5).

*Histochemical Determination of Kidney Function with Ferrocyanide.*—The modified Gersh ferrocyanide technique was applied to animals in the groups described above. In the first stage when the tubules were filled with casts ferrocyanide was found within all the glomerular spaces and within the lumina of the proximal convoluted tubules, while the distal portions of the tubular system which were filled with casts contained no ferrocyanide. The few dilated tubules with flattened epithelium which were free of casts contained ferrocyanide within their lumina. No staining of tubule cells was seen (Fig. 6). This distribution of ferrocyanide—in the glomerular spaces and lumina of only the proximal portions of the tubular system—resembled that found in animals in which an acute hydronephrosis had been produced by ligation of the ureters 24 hours before. It is of interest that filtration of fluid through the glomerular capillaries continues despite acute obstruction of the tubules or of the ureter. In the kidneys of dogs studied 3 to 4 days and thereafter following exposure to arsine or injection of hemoglobin, the ferrocyanide was visualized in all of the glomerular spaces and in the lumina of the dilated tubules but not in the collapsed tubules. The absence of ferrocyanide in these collapsed tubules suggested that there was no flow of urine through them although no obstructing casts were seen. The large dilated tubules seen in the late stages (dog 16-45, Fig. 5) contained ferrocyanide and were presumably the only functioning tubule systems.

*Properties and Identification of Pigment of the Casts.*—Thick frozen sections of unfixed kidney were mounted on glass slides without contact with water and then kept in a humid chamber to prevent dehydration. The yellow-brown casts were teased out by micro dissection. They were jelly-like in consistency;



TEXT-FIG. 6. Spectrophotometric absorption curves of dissolved casts from kidney of dog 16-97, examined 4 days after exposure to arsine. Wave length given on abscissa and light transmission in terms of  $2 + \log_{10} d$  ( $d = \log \frac{I_0}{I}$ ) on ordinate. By this method of plotting, the shapes of the curves are independent of the concentration of pigment (19). Curve 1, absorption spectrum, pH 5.2, acid methemoglobin. Curve 2, absorption spectrum, pH 9.0, alkaline methemoglobin. Curve 3, absorption spectrum, following addition of 0.1 per cent NaCN to solution at pH 7.7, cyanmethemoglobin.

TABLE VI  
*Identification of Pigment Casts*

Dog No.	Treatment	Interval post exposure or injection	Absorption spectrum of dissolved cast material	Qualitative test for methemoglobin
		<i>hrs.</i>		
16-98	Arsine	15-18	Oxyhemoglobin	Positive
17-07	"	20	Methemoglobin	—
17-22	"	21	—	Positive
17-23	"	23	—	Positive
16-91	"	24	Oxyhemoglobin	Positive
16-93	"	48	—	Positive
16-94	"	48	—	Positive
16-90	"	48	Methemoglobin and oxyhemoglobin	—
16-92	"	72	Methemoglobin	Positive
16-97	"	96	Methemoglobin	Positive
17-16	Injection of methemoglobin	2	—	Positive

TABLE VII

*Correlation of Number of Casts, Histological Changes in Proximal Convoluted Tubules, and Evidences of Impairment of Renal Function*

## A. Dogs exposed to arsine

Dog No.	Days post exposure	Plasma		Average No. casts per field		Renal tubular "hemoglobin" droplets	Epithelium necrosis
		Urea N	Creatinine	Cortex	Medulla		
		mg./100 cc.	mg./100 cc.				
15-83	$\frac{1}{2}$	36	—	12	0	+	0
15-38	1	26	—	7	20	+	0
15-39	1	35	—	4	10	+	0
15-90	1	47	—	35	10	+	0
15-02	1	55	—	80	35	+	0
15-22	1	56	—	30	12	+	0
14-46	1	59	—	40	55	+	0
15-84	1	93	—	60	80	+	+
14-96	1	93	—	80	90	+	+
15-13	1	105	—	12	65	+	+
15-51	$1\frac{1}{2}$	24	—	30	15	0	0
15-01	$1\frac{1}{2}$	42	—	2	30	+	+
14-79	2	86	—	20	0	+	0
16-86	2	96	2.2	90	10	+	+
16-94	2	—	4.6	160	95	0	+
16-93	2	—	8.9	90	200	0	+
15-41	$2\frac{1}{2}$	75	—	12	0	0	0
14-36	3	25	—	6	5	0	0
14-54	3	31	—	0	2	0	0
15-04	3	41	1.3	10	20	0	0
16-92	3	123	—	150	140	+	+
16-80	3	123	3.3	25	35	0	+
16-97	4	91	—	50	50	0	+
15-98	4	132	—	50	10	0	0
16-83	4	257	—	40	70	+	+
17-08	4	—	7.4	200	100	0	0

## B. Dogs injected with methemoglobin

16-64	1	—	1.1	10	12	0	0
16-63	1	—	4.6	18	100	+	+
15-70	2	8	—	5	12	0	0
15-59	2	185	—	180	10	+	+
16-57	3	—	3.9	120	90	+	+
16-30	3	92	—	5	30	0	0
16-12	4	183	—	2	10	0	0
16-41	5	185	—	3	85	0	0
15-65	5	253	—	3	35	0	0
15-44	5	261	—	3	75	0	+

they retained their shape when manipulated gently and could be cut up into discrete segments. Their solubilities in buffer solutions of varying pH were determined by dropping the free casts into the buffer solutions or by adding a drop of buffer solution to the frozen section and observing the dissolution of the casts under the microscope. The casts observed in the kidneys of animals 18 hours to 4 days following exposure to arsine or injection of hemoglobin or methemoglobin were dissolved rapidly by solutions of pH 5.2 or below, or 7.6 or above, while they were dissolved slowly by solutions of pH 6.7 to 7.0.

The absorption spectra of the solutions of the pigment casts were determined with a Beckman spectrophotometer. In the specimens obtained from kidneys with large number of casts, the characteristic absorption curves of methemoglobin were found (Text-fig. 6). By suitable change of pH the spectrum of acid or alkaline methemoglobin was observed, and the typical shift to the curve of cyanmethemoglobin was seen when NaCN was added. An occasional preparation from a kidney in which casts were few in number gave the curve of oxyhemoglobin, probably due to the residual red blood cells in the section. Qualitative histochemical tests were made on the casts *in situ* by exposure of the frozen sections to vapors of HCN or addition of a drop of NaCN solution while the casts were observed through the microscope. In all instances in which this was done, the casts developed the characteristic orange-red color of cyanmethemoglobin (Table VI).

*Correlation of Number of Casts and Degree of Impairment of Kidney Function.*—The number of casts per unit area in both the cortex and medulla were determined. Within the field used (2.2 sq. mm.) the total number of tubule cross-sections was found to average approximately 500 in the cortex and 400 in the medulla. The yellow-brown casts only were counted since these were assumed to be the ones capable of obstructing the tubules. In Table VII are given the data on the kidneys of dogs exposed to arsine and those given intravenous injections of methemoglobin and hemoglobin solutions. The cast count is the average of several fields. The data are necessarily approximations but they indicate that the degree of impairment of kidney function as measured by the elevation of plasma urea and creatinine was greatest in those animals in which the casts were most numerous. Conversely, when few casts were found, there was little evidence of impairment of kidney function. In four instances a moderate degree of renal insufficiency was present with only a small number of casts (30 to 40 per field). In these animals, no anatomical basis for the impaired renal function was found.

#### DISCUSSION

The experiments reported here indicate that the early impairment of renal function observed in dogs after extensive intravascular hemolysis due to arsine poisoning or after intravenous injection of solutions of methemoglobin and hemoglobin can in large part be explained by obstruction to flow of urine



through the renal tubules. By means of the ferrocyanide histochemical method it was demonstrated that filtration through the glomerular capillaries was continuing since ferrocyanide was found in the glomerular spaces. No evidence was found that the tubule cells were sufficiently injured at this stage to permit extensive back diffusion of the glomerular filtrate since staining of the tubule cells by ferrocyanide was not seen. On the other hand, in animals in which renal injury was produced by mercuric chloride, the tubule cells were found to be permeable to ferrocyanide. The absence of ferrocyanide in the distal portions of the tubular system in the hemoglobinuric animals can be explained on the basis of obstruction to flow of urine through the renal tubules. By means of micro dissection of nephrons Oliver (20) has also concluded that obstruction to the flow of urine through the tubules is the basis for impaired renal function in hemoglobinuria and other states in which protein solutions of high viscosity are found within the tubule. It must be kept in mind that in the dog, impairment of kidney function as the result of hemoglobinemia occurs only with high concentrations of plasma pigment. When methemoglobin concentrations in the plasma of 1 to 2 gm. per 100 cc. were produced, severe progressive renal injury was found only when the urine flow was reduced at the time of the injection of methemoglobin. These conditions would obviously predispose to the development of tubular obstruction because of the high concentration of heme pigment in the urine. Under other conditions in other species of animals the findings might not be the same.

Previous workers have suggested that obstruction of the renal tubules is the important factor in the renal injury produced by hemoglobin or its derivatives and have postulated that the obstruction is due to the precipitation of an insoluble heme pigment in the tubules; *viz.*, hematin (1). In our studies, the casts dissected from the tubules in freshly frozen unfixed sections of kidney were found to be composed chiefly of methemoglobin. Solubility studies of the cast material indicated that the casts were soluble in buffer solutions over a wide range of H ion concentration, but were least soluble at pH 6.7 to 7.0, which is approximately the isoelectric point of hemoglobin and methemoglobin. Obstruction to flow of urine was not due to the precipitation of insoluble hematin in the lumen of the tubule, but apparently resulted from the viscosity of the concentrated solution of hemoglobin or methemoglobin. If the resistance to flow of such a solution through the tubules were greater than the pressure gradient available for propelling the solution through the renal tubules *i.e.* the blood pressure in the glomerular capillaries plus the forces resulting from reabsorption of water in the tubules, cessation of urine flow could be explained. Once the tubules are obstructed by this viscous protein solution it is unlikely that urine flow can be restored by the administration of water or electrolyte solutions.

The greater degree of depression of renal function produced by injections of

methemoglobin than that resulting from administration of oxyhemoglobin and the effect of acidosis in increasing the injurious action of methemoglobin cannot be explained on this basis. The increased toxicity of methemoglobin was thought by Corcoran and Page (21) to be due to the more rapid formation of hematin from methemoglobin than from oxyhemoglobin and the effects of acidosis were considered to be due to the influence of urine pH on the conversion of methemoglobin to hematin. Our studies, however, do not show any evidence of the formation of detectable amounts of hemochromogen or hematin in the lumina of the tubules either after intravascular hemolysis or after the intravenous injection of methemoglobin. The possibility that methemoglobin might be filtered more readily through the glomeruli than hemoglobin and thus be found in greater concentration in the tubular lumen was considered, but comparison of renal clearances of hemoglobin and methemoglobin does not show any consistent differences (22). Histological evidence of necrosis of renal tubule cells was found in these studies as in many of the earlier reports of renal injury due to hemoglobin (4, 5). The maximum degree of necrosis was found 1½ to 2 days after the infusion and unquestionably served as an additional factor which in combination with the increase in resistance of urinary flow due to the viscosity of the tubular contents could account for the persistent impairment of renal function seen in these animals. Hemoglobin or methemoglobin (these pigments stain alike with the benzidine or alum-hematoxylin method) is found within the cells of the proximal convoluted tubules following their filtration through the glomeruli. Hemoglobin is oxidized to methemoglobin in both the plasma and urine, but this oxidation is more complete in the urine particularly when obstruction to urine flow exists. Methemoglobin may act as an oxidant and a possible mode of its toxic action may be the catalysis of the oxidation of sulfhydryl groups. There is evidence that the renal tubular epithelium is extremely susceptible to agents which combine with or oxidize sulfhydryl groups (23).

Renal ischemia has been considered as a possible cause of the depression of renal function in conditions associated with hemoglobinuria. A decrease of kidney volume has been observed immediately following injection of hemoglobin solutions and interpreted as an indication of reduction of renal blood flow (7, 8). Our direct measurements of renal blood flow have not confirmed this interpretation. The reduction of diodrast clearance seen at a later stage of renal injury following injection of methemoglobin (18) may be due to the presence of many non-functioning nephrons rather than to diminution of total renal blood flow.

#### SUMMARY

Severe and persistent impairment of kidney function has been produced in dogs by intravascular hemolysis due to arsine, or by the intravenous injection of solutions of dog hemoglobin and methemoglobin.

The kidneys of these animals have been examined by the usual histological methods and also by means of the ferrocyanide histochemical method to determine the pathogenesis of the renal injury. These observations indicate that obstruction to flow of urine through the renal tubules is an important factor in the early reduction of kidney function. The material filling the lumina of the renal tubules was found to be chiefly methemoglobin in concentrated solution of gel-like consistency. No evidence of formation of a pigment insoluble at the pH of the urine such as hemochromogen or hematin was found. The cessation of urine flow is most readily explained by the increased viscosity of the tubule contents.

The intravenous administration of methemoglobin was found to produce more severe renal injury than the injection of equal amounts of oxyhemoglobin. Necrosis of the proximal convoluted tubule cells was present as a late lesion in animals injected with methemoglobin, large amounts of hemoglobin, or following extensive intravascular hemolysis. Such injury is probably a contributing factor in the persistent severe depression of renal function seen in these animals. Following disappearance of most of the intratubular pigment, a large number of collapsed tubules lined by hemosiderin-filled cells were found. The ferrocyanide histochemical studies indicated that these represented non-functioning nephrons although no obstructing intratubular material was present.

Direct measurements in two animals failed to reveal any reduction of renal blood flow following the injection of methemoglobin in amounts sufficient to produce renal injury.

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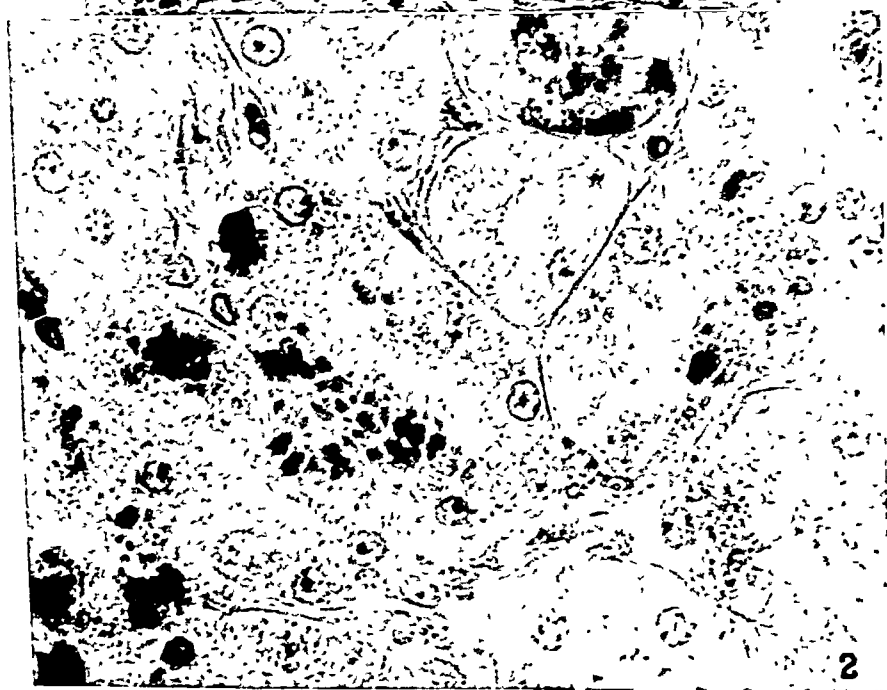
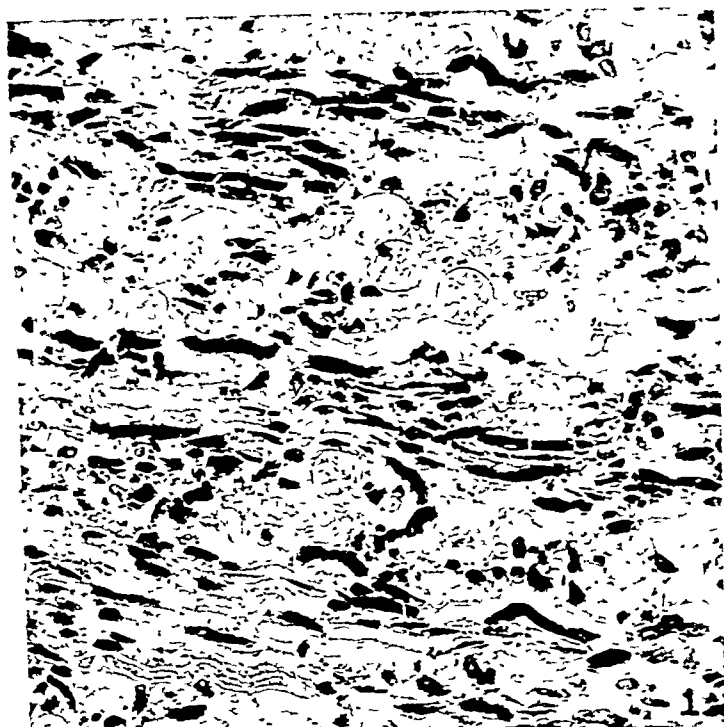
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#### EXPLANATION OF PLATES

##### PLATE 32

FIG. 1. Dog 14-63. Died approximately 24 hours after exposure to arsine. Extensive deposition of hemoglobin or its derivatives within the tubules, chiefly distal convoluted and collecting tubules. Iron-alum-hematoxylin.  $\times 52$ .

FIG. 2. Dog 14-79. Died 2 days after exposure to arsine. Plasma urea N 86 mg. per 100 cc. "Hemoglobin" droplets within epithelium of the proximal convoluted tubules. Iron-alum-hematoxylin.  $\times 575$ .

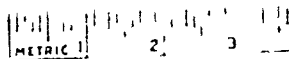


### PLATE 33

FIG. 3. Dog. 14-79. Pyknosis of some of the cells of ascending limb and distal convoluted tubules adjacent to pigment casts within the lumen. Hematoxylin and eosin.  $\times 355$ .

FIG. 4. Dog 16-97. Examined 4 days after exposure to arsine. Blood NPN 91 mg. per 100 cc. Transverse and longitudinal sections of the kidneys showing the radial arrangement of the zones of persistent pigment casts in cortex and medulla.

FIG. 5. Dog 16-45. Sacrificed 10 days after injection of methemoglobin solution. Plasma urea N 139 mg. per 100 cc. Plasma creatinine 6.2 mg. per 100 cc. Irregular dilatation of some of the tubules with zones of collapsed tubules. Hematoxylin and eosin.  $\times 100$ .



3

4

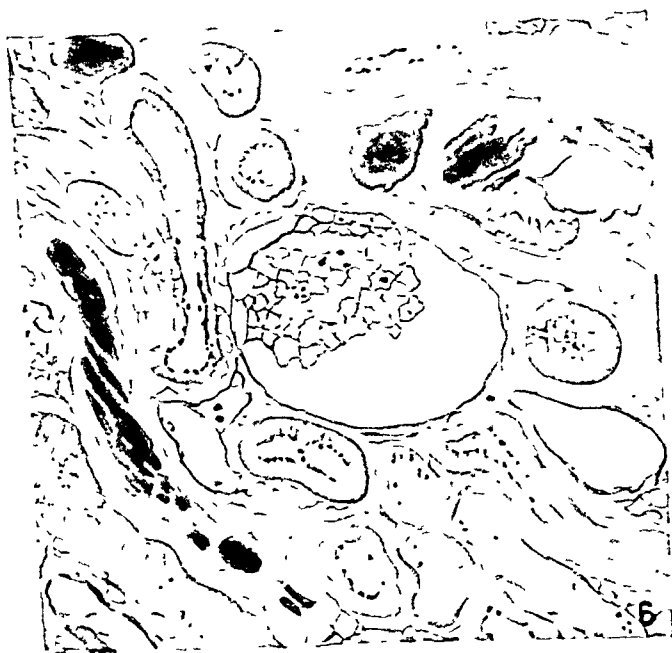


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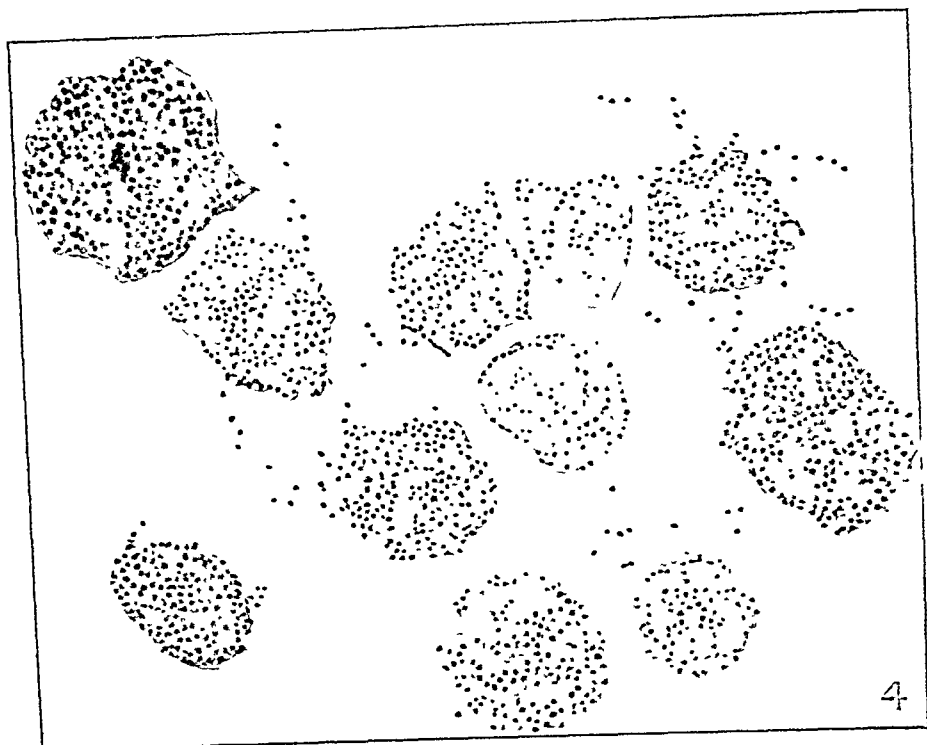
PLATE 34

FIG. 6. Dog 16-63. Sacrificed 1 day after injection of methemoglobin. Plasma creatinine 4.6 mg. per 100 cc. Section of kidney prepared by modified Gersh method (14). The drawing shows the blue precipitate of ferric ferrocyanide in Bowman's space and the lumina of the proximal convoluted tubules but not in the distal convoluted tubules that contain pigment casts. The tubular epithelium is not stained. The presence of ferrocyanide in the lumen of some of the blood vessels and lymphatics is noted.









(Oliver *et al.*: Origin of heparin)

*This plate should be substituted for the original Plate 8 which appeared in Vol. 86, No. 2, August 1, 1947, and showed faulty register of the colors*

## PLATE 9

FIGS. 6 to 12. Photographs of living unstained tumor mast cells taken by phase contrast microscopy. Under such circumstances the diaphragm is fully open and the maximum contrast occurs only at the exact focus. Objects out of focus appear with diffraction rings (*vide* floating extracellular granules in Fig. 12). This means that the variations in the density of the intracellular particulate material are not the result of the common optical artifacts produced by cut-down illumination and out of focus examination, but that the considerable differences in appearance are optically real.  $\times 2860$ .

FIG. 6. Living unstained cell of the immature type. The cell has been slightly flattened by pressure. The cytoplasm is filled with particulate material presenting three optical appearances; densely black discrete granules, similar black granules with optically clear centers, and diffusely scattered greyish material in granular form. This last is in the same optical plane of focus as the sharply defined black granules.

FIGS. 7 to 11. Living mast tumor cells from the same specimen. In these preparations the cells were floating in fluid, so the cytological detail is less clear because of their relatively great thickness. All show diffusely distributed greyish particulate matter among which the dense black granules are scattered in irregular clumps.

FIG. 12. A living mast cell from a mature tumor. The cytoplasm is crowded almost to bursting with dense black granules and there is very little greyish particulate matter.

# STUDIES ON THE MECHANISM OF ADAPTATION OF INFLUENZA VIRUS TO MICE

By GEORGE K. HIRST, M.D.

(From the Laboratories of the International Health Division of The Rockefeller Foundation, New York)

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Viruses are often adapted to new hosts, but as yet little is known of the mechanisms involved in the process. It is commonly assumed, and in many cases may be true, that on initial passage in a new host a virus grows poorly and few of the numerous particles inoculated survive. The survivors are presumably variants, which differ from the rest in having a greater capacity for multiplication in the new host. By serial passage these variants are selected and fostered, and in the beginning they may have no detectable effect, thus giving rise to the term blind passage. Complete adaptation can be assumed to be due to the emergence of further variants and their selection, until a strain capable of achieving a high titer is obtained. These latter stages may be accompanied by the appearance of pathological lesions or by inability of the host to survive. If such is the case it is not necessary to assume that the occurrence of lesions is correlated with any other change in the virus except its capacity to multiply in host cells.

The adaptation of influenza virus to the lung of the mouse, reported in this paper, may be a special case, but in any event it is an interesting one and necessitates some other interpretation of the adaptive process.

It has been the usual experience that a fresh human strain of influenza virus can be readily adapted to mice after a few ferret passages. Five to twenty mouse passages enhance the virulence to such a degree that lung suspensions will kill in dilutions of  $10^{-2}$  to  $10^{-6}$ . The same strains will cause lung lesions in dilutions about two logs beyond the lethal end point. In the absence of any means of detecting the presence of influenza virus other than by lethal or lung consolidating effect, it has not been possible to tell what amounts of virus there might be in the mouse lung during the early passages, and it has seemed possible that the increased pathogenicity of the virus with passage might be correlated with increased capacity to multiply in the lung.

With the development of the *in ovo* method of titrating influenza virus (1), a very delicate means of virus measurement became available which enables the investigator to follow the course of virus growth in the mouse lung during a period of adaptation and thus to correlate ability of the virus to multiply with changes in mouse virulence. The method is especially suitable, since all the evidence points to the fact that the *in ovo* titer of the virus is quite independent of the degree of mouse adaptation.

A second aspect of the study reported here was the examination of virus strains for change in antigenic pattern in the course of adaptation to the mouse. Practically all the descriptions of antigenic differences among influenza A and influenza B virus strains consist of comparisons of strains which have been repeatedly passed in mice before they have been tested. It has never been certain whether or not many of the differences found were present in the original organisms or were produced by passage in animals. The work on strain adaptation described below offered the possibility of examining such variation.

### *Methods*

*Virus Strains.*—The strains used in the present study were Ala. 41, Kil. 41, N.Y. 43, and Sinai 45. The first three are strains of influenza A and the last is a strain of influenza B. Ala. 41 and Kil. 41 were isolated from patients in Alabama during the influenza A epidemic of 1940–41, N.Y. 43 was obtained in New York City during the epidemic of December, 1943, and strain Sinai 45 was isolated from the lung of a patient who died in Mount Sinai Hospital, New York City, of acute hemorrhagic tracheobronchitis during the epidemic of influenza B in November, 1945.<sup>1</sup> All these strains were initially isolated by inoculation of garglings or lung suspension into the amniotic sac of the developing chick embryo (2).

*Mouse Passages.*—The adaptation of the strains to mice was carried out by the usual methods. Suspensions were inoculated intranasally in 0.05 cc. amounts into six mice, which were sacrificed in 3 or 4 days. The lungs of the mice were ground in 10 per cent normal horse serum broth and passed in series at a 10 per cent concentration. As virulence increased, inocula of greater dilution were used. Titration of passage material in mice and *in ovo* was usually done immediately after preparation but sometimes only after storage at  $-72^{\circ}\text{C}$ . With passage of strain N.Y. 43, mouse lungs were perfused with buffer before removal. Hemagglutinin titrations were done on perfused lung suspensions, using a pattern technique like Salk's (3). For control purposes similar titrations were done simultaneously on normal mouse lung.

*In Ovo Titrations.*—The titration of mouse lung suspensions in the allantoic sac of developing chick embryos was carried out by methods already described (1). The virus was diluted in tenfold steps, and each dilution in the amount of 0.1 cc. per egg was inoculated into six eggs. After incubation for 48 hours the eggs were placed in a refrigerator overnight. The allantoic fluids were then tested individually for agglutinins, and the 50 per cent egg infective titer was calculated in the usual manner (4).

*Titrations in Mice.*—For these titrations the virus was diluted in tenfold steps and each dilution in 0.05 cc. amounts was inoculated intranasally into six mice. All mice dying in the first 10 days were examined for the presence of specific lung lesions; the remainder were sacrificed at the end of 10 days and the extent of their lesions was recorded. The 50 per cent mortality end points and the 50 per cent lesion end points were calculated (5), the latter being based on the presence of one-plus or greater lung lesions.

*Cross-Tests for Strain Differences.*—The mouse passage strains to be tested were grown in the allantoic sac of chick embryos, a considerable amount of virus being prepared. Ferrets were inoculated with a  $10^{-3}$  dilution of this material, and the serum obtained 2 weeks later was used for cross-tests. Agglutinin inhibition titrations were performed with the aid of a densitometer (6), and all cross-tests were made at the same time (7). The results, as heretofore, have been expressed graphically as a ratio of the homologous titer to the heterologous titer with any given serum.

<sup>1</sup> This specimen was obtained from the pathology service of Mount Sinai Hospital through the courtesy of Dr. Klemperer.

## EXPERIMENTAL

*Correlation of Virus Multiplication with Enhancement of Virulence in the Mouse Lung*

Influenza A strains which have been isolated in chick embryos can be established directly in mice without any difficulty. This has been true of the strains of 1940-41 and 1943-44. The first strain selected for study of adaptation was Ala. 41, which was isolated by amniotic inoculation and subsequently went through four allantoic passages. The allantoic fluid of the fifth egg passage was titrated *in oro* and had an infectious titer of  $10^{-7.5}$ . This material was

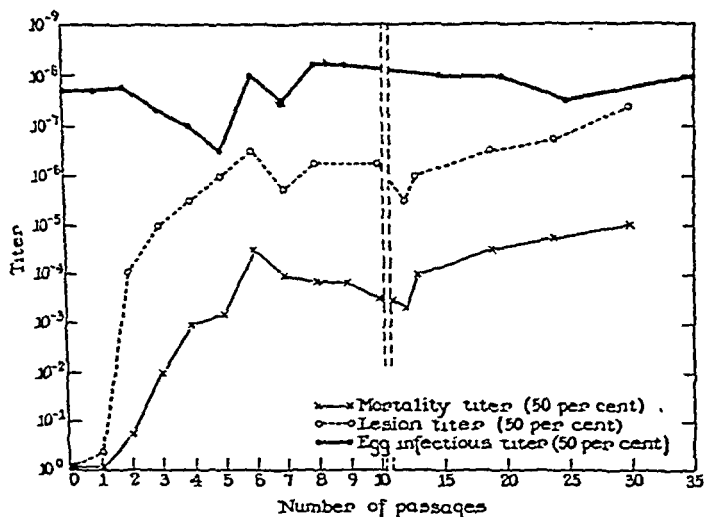


FIG. 1. The adaptation of influenza A strain Ala. 41 to mice. Curves of titer of egg infectivity, mouse lesion titer, and mouse mortality titer of a strain of influenza A virus in different stages of the adaptation to the lungs of mice by serial passage. Zero passage indicates titrations of the allantoic fluid starting material, passage one indicates titrations on lungs of the first mouse passage on the 3rd day, etc.

inoculated intranasally into six mice. After 3 days the mice were killed, and the lungs were ground and passed in 10 per cent concentration to six more mice, and so on in series. Each mouse lung preparation and the original allantoic fluid were titrated both *in oro* and in mice, with results shown graphically in Fig. 1. In this chart zero passage refers to the titration of the allantoic fluid starting material, passage one indicates titration results of the first mouse passage, etc.

Although the initial inoculum was large in terms of egg infectious particles it did not cause any deaths in mice, and only negligible lung lesions. It was very surprising therefore to find that these first passage lungs, most of which appeared perfectly normal, contained very large amounts of virus. In terms

of egg infectivity, mouse lungs of the first passage contained as much virus as did those of subsequent passages when the mouse virulence increased so much that dilutions of  $10^{-4}$  and  $10^{-5}$  of mouse lung were able to kill. This level was achieved by the sixth passage, and virulence was maintained without further increase through many subsequent generations. The lesion end point was fairly consistently two dilutions higher than the mortality end point. While there were minor irregularities in the *in ovo* titrations, in general the egg infective titer remained between  $10^{-7}$  and  $10^{-8}$  throughout the whole period of adaptation and showed no marked tendency to increase with passage.

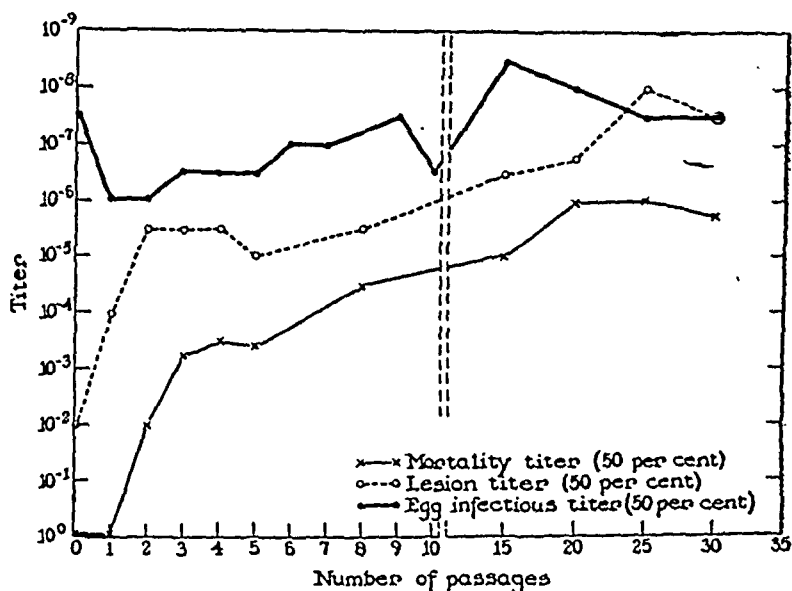


FIG. 2. The adaptation of influenza strain Kil. 41 to mice. The curves are similar to those shown in Fig. 1. The virus being tested is from the same epidemic and in its egg-isolated form was similar antigenically to the strain Ala. 41, of Fig. 1.

Fig. 2 shows the results of a similar effort with the closely related strain Kil. 41 from the same epidemic. With this strain there was some tendency for the allantoic titers to increase between the tenth and the thirty-fifth mouse passage, but no change took place during the early stages when the mortality and lesion titers were rapidly rising. As in the case of strain Ala. 41, there was a high titer of virus in the initial lung passage with very little evidence of gross lesions. It can also be noted that with neither of these strains was the rise in mortality with passage sharply stepwise; it was steady for about five passages before levelling off at a lower rate of increase.

The results of another passage series with strain N. Y. 43 (type A 1943) are shown in Table I. There was no marked difference from the previous results. While the mortality titer rose from less than  $10^0$  to  $10^{-5.2}$  in four passages, the



*in oro* titer remained nearly constant for thirty passages. In this series an attempt was made to correlate the hemagglutinin titer in the lungs with the *in oro* titer, and all lungs were perfused before removal from the mouse to eliminate murine red cells from the suspension. The hemagglutinin tests were done by the pattern method in order to increase the sensitivity, and suspensions were tested with both guinea pig and chicken red cells. The results were not entirely consistent; but two trends may be noted: (1) The chicken cell agglutinin titer did not increase with passage, while the guinea pig titer increased markedly; and (2) the guinea pig/chicken cell titer ratio of the starting virus was 1, but during passage was usually greater than 1 and increased to 128 on the twenty-eighth passage. It might be inferred from this that the virus

TABLE I  
*Adaptation of Strain N.Y. 43 to Mice by Serial Passage*

Passage No.	<i>In oro</i> titration	Titration in mice		Hemagglutinin titer	
		Mortality	Lesion	Chicken cells	Guinea pig cells
0	$10^{-3.2}$	$10^0$	$10^{-2.5}$	1:320	1:320
1	$10^{-7.4}$	$10^{-2.5}$	$10^{-5.2}$	1:10	1:40
2	$10^{-6.7}$	$10^{-5.0}$	$10^{-5.0}$	1:10	1:40
3	$10^{-6.3}$	$10^{-4.0}$	$10^{-5.0}$	1:10	1:160
4	$10^{-6.3}$	$10^{-5.3}$	$10^{-5.0}$	1:10	1:10
5	$10^{-6.3}$			1:10	1:20
10	$10^{-7.0}$			1:10	1:10
15	$10^{-6.5}$			1:10	1:160
20	$10^{-7.5}$			1:10	1:40
25	$10^{-7.0}$			1:10	1:20
28	$10^{-6.8}$			1:10	1:1280
30	$10^{-7.0}$			1:20	1:640

(originally D form) was becoming with passage more like the O form of Burnet and Bull (8), although this seems at variance with the contention of these authors that the latter multiplies poorly in mice compared with the D form.

A passage series was also started with the type B strain Sinai 45. This strain, in common with other examples from this epidemic (9), did not adapt quickly to the allantoic sac, and even after egg adaptation it could not be established in mice. Virus persisted detectably for only one or two passages and then disappeared. After passing the egg-isolated strain through three ferrets it became possible to infect mice regularly in passage series. In the ferret the virus was obtainable only from the turbinates. In the initial mouse passage no lesions were present (*i.e.* 10 days after inoculation); after fifteen passages, only small and scattered lesions were seen and there were no deaths among the animals. Allantoic titrations of passage lungs have shown a max-

imum titer of  $10^{-5}$ , but the results have not been consistent, presumably because of the poor allantoic adaptation of the strain. This passage series is being continued.

### *The Development of Strain Differences with Mouse Passage*

Magill and Francis (10, 11) and Smith and Andrewes (12) demonstrated with a large number of examples that influenza A strains, even from patients in the same epidemic, often showed marked antigenic differences one from the other. Acceptance of these differences for human virus necessitates the assumption that influenza virus undergoes continual radical changes in antigenic pattern with human to human passage or that epidemics arise commonly from multiple foci and are due to heterogeneous agents of the same type. There is another interpretation of these observations however which seems equally likely, namely that the strain variations found did not exist in the original human strains but were the result of the adaptation of these strains to mice. This does not seem like a remote possibility, since mouse adaptation induces marked behavior changes in strains, and it is by no means unlikely that these are accompanied by antigenic pattern changes as well.<sup>2</sup>

With the advent of the agglutination test, another method became available for making strain comparisons, which has been shown to be adequate for detecting minor antigenic differences. A reexamination of the strain difference problem with this *in vitro* method completely confirmed the findings of earlier workers when the same (mouse-adapted) strains were compared (7). However, when a large number of strains from the 1940-41 epidemic of influenza A were cross-tested it was found that viruses isolated directly in chick embryos did not differ from one another at all, while two strains isolated in ferrets and mice differed from each other and from all the egg-isolated examples. These findings also suggested that mouse adaptation might be at the bottom of the strain difference problem, and further data from two later epidemics (9) has reenforced this view. To furnish a direct test of this possibility, two strains (Ala. 41 and Kil. 41) identical in their egg-isolated form have been studied.

Each strain was started from throat washings inoculated into ferrets. Six ferret passages were necessary before they could be carried in mice. Virus could be obtained only from ferret turbinates. It was maintained in mice by passage at 4-day intervals. After thirty mouse passages of Ala. 41 (Ala. M) and twelve of Kil. 41 (Kil. M), antisera were prepared against these strains in ferrets, and they were cross-tested with their non-mouse-adapted counterparts (Ala. E and Kil. E). The results of this cross-test are shown graphically

<sup>2</sup> Magill and Francis (11) discussed this possibility and tested for antigenic changes in PR8 virus between the thirtieth and the two hundred eighty-fifth mouse passage, but found none. The necessity of using a mouse-virulent virus for cross-testing did not permit the examination of unadapted virus of early passages.

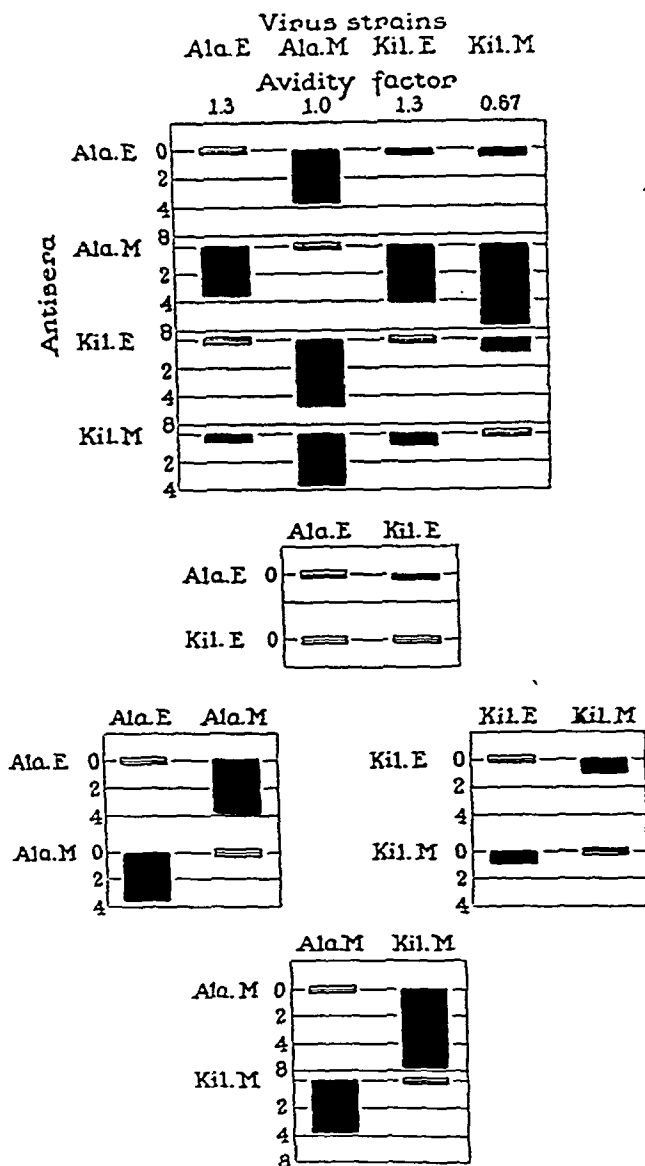


FIG. 3. Antigenic comparison between two influenza strains isolated in chick embryos and their mouse-adapted counterparts; results of cross-tests between the various strains and their ferret antisera by agglutination inhibition. The homologous value is by definition zero; the black bars indicate how much less the titer was with heterologous than with homologous strains and their height is an indication of the degree of antigenic shift.

An empiric correction for the avidity factor is given at the top of the figure. This factor renders the differences found more nearly equal on both sides of a cross-test and is probably made necessary by varying states of aggregation in different virus suspensions. The complete test given in the upper figure is broken down below to facilitate specific comparisons.

in Fig. 3. Small corrections have been applied to the results to make them more reciprocal. The rationale of these has been previously described (7), but the essential results can be demonstrated without this factor. As may be seen from Fig. 3 the sera prepared from strains Ala. E and Ala. M inhibit the agglutination of the heterologous strains only one-fourth as well as that of the homologous strains. This result is highly significant of a true strain difference as measured by this method. The mouse strain Kil. M had had only twelve passages in mice and differed from strain Kil. E by less than twofold, just on the borderline of significance. Both strains in the egg-adapted form (Ala. E and Kil. E) are identical within the limits of this test, but the mouse-passage derivatives of these two strains differ from each other to a greater degree than either one differs from its egg-passage antecedent. Examination of these strains at other stages of mouse adaptation gave confirmatory results.

Thus the *in vitro* type of cross-test showed that two strains of influenza virus shifted in antigenic pattern with mouse passage. The strains in their egg-isolated form were identical, and with mouse passage they not only deviated from their original pattern but deviated from each other.

#### DISCUSSION

The results of experiments with mouse lungs which had been inoculated with egg-adapted influenza A virus make it clear that a full-blown influenza infection with maximum virus multiplication may occur in the mouse without any gross evidence of a pathological process. In this case the enhancement of virus virulence with passage is not due to an increasing ability of the agent to grow in the lung. Rather, the egg-adapted type of virus seems to be gradually replaced by an entity possessing the same growth potentialities but new and different pathogenic qualities. The pathogenic virus, in order to predominate, must have a more rapid growth rate than its innocuous predecessors. Preliminary experiments have indicated that both forms reach the same maximum titer in the lung, but the pathogenic strain reaches the maximum a little faster. The way in which the mortality titer rises, slowly and steadily for four or five passages, is also consistent with the gradual predominance of a pathogen which grows at a slightly faster rate. The regularity with which mouse virulence is attained with egg-passage strains suggests that the lethal strain may be produced with a fairly high frequency and consistency. The sharp initial rise in virulence levels off at  $10^{-4}$  to  $10^{-5}$ , and subsequent increases, possibly due to other variants, are less predictable.

Just how comparable results of serial passage of influenza B virus may be cannot be stated at present since in forty passages of this virus no appreciable enhancement of virulence has occurred, and the relatively poor adaptation to the allantois makes the significance of the titrations less certain.

While the behavior of red cell agglutinins in passage mouse lungs was notable

for its extreme variability, there was nevertheless a tendency toward the development of guinea pig cell agglutinins and not chicken cell agglutinins. This finding and those of Burnet and Bull (8) on freshly isolated human strains suggest that mammalian cells may have some characteristics in common in respect to agglutinability by certain forms of influenza virus. Maintenance of the virus in man (or in the mouse) fosters the development of the mammalian type of virus agglutinin, while with chick embryo growth the avian agglutinin is very rapidly developed *without*, however, any loss of affinity for mammalian cells. This can be described as only a tendency, since there are numerous examples of laboratory strains which have been carried in mammals only but which possess high chicken cell agglutinins.

The change in antigenic pattern on passage of a strain through mice is of interest mainly in that it removes a good deal of the foundation on which the knowledge of antigenic differences began. We now have evidence that with mouse passage two similar strains will not only change from their original pattern but will deviate from each other. This source of error has in no sense been eliminated from the original, and much of the later, work on this subject. Further evidence will be given in the succeeding paper on the homogeneity of strains in various epidemics, and the implications of these findings will be developed in the light of more complete evidence. As has been stressed earlier the occurrence of antigenic differences with mouse passage is by no means unexpected, and the fact that the shift in antigenic pattern does not follow a definite direction but diverges in different series is also in line with what might be expected from the selection of chance variants. The degree of antigenic difference produced was of the same order of magnitude as that found by Magill and Francis (11) but is definitely less than that described by Smith and Andrewes (12). Why the English workers found such large variations is not clear; antigenic discrepancies of similar magnitude have never been found in the United States. It should be emphasized that the description of antigenic shifts by animal passage does not rule out the occurrence of differences in human strains but merely requires added proof of such differences, which at the present time is not sufficient where strains from a single epidemic are concerned.

#### SUMMARY

1. When strains of influenza A virus which have been isolated in chick embryos are introduced into the mouse lung, the virus multiplies readily and achieves initially a titer which is as high as is ever obtained, even after repeated passage. The high initial titer of virus may be unaccompanied by any lethal or visible pathogenic effects; but with four or five mouse passages the agent becomes lethal in high titer and causes extensive pulmonary consolidation, though its capacity to multiply in the lung has not increased. In one

example the adaptation to mouse lung was accompanied by increasing capacity to agglutinate guinea pig red cells without a corresponding increase in agglutinating power for chicken cells. Influenza B virus, in preliminary tests, did not behave in a similar fashion.

2. The adaptation of influenza A virus to mice is accompanied by changes in antigenic pattern, as detected by cross-tests with the agglutination inhibition method. Two strains, initially similar, with passage, changed in pattern along divergent paths so that they became not only unlike the parent strains but unlike each other. This finding has important implications for the interpretation of the strain difference problem in human influenza.

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# COMPARISONS OF INFLUENZA VIRUS STRAINS FROM THREE EPIDEMICS

By GEORGE K. HIRST, M.D.

(From the Laboratories of the International Health Division of The Rockefeller  
Foundation, New York)

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In the course of numerous field studies of influenza made in the past few years, a considerable amount of information has been accumulated on the behavior of virus strains isolated at different times. This information is of some immediate practical value, since much of it concerns difficulties encountered in virus isolation, which are important in making a laboratory diagnosis. The observations cover the characteristics of influenza A strains isolated during the outbreaks of 1940-41 and 1943-44 and of influenza B virus isolated in 1945-46. A number of the strains were tested for antigenic differences, the results of which may have some bearing on the selection of viruses for human vaccination.

## *Materials and Methods*

*Virus Strains.*—Four strains of influenza A virus were used. Strain 1741 was obtained from Dr. E. R. Rickard, then at Minneapolis, Minnesota, and is herein referred to as the Minn. 43 strain. Strain ICE49 was obtained from Dr. W. H. Hale and is referred to as the Iowa 43 strain. The Martin strain of influenza A virus was obtained from Dr. J. E. Salk and is called the Mich. 43 strain. The Olson strain, received from Dr. M. D. Eaton, will be referred to as the Calif. 43 strain. All of these strains were isolated during the outbreak of influenza A occurring between mid-November, 1943, and February, 1944. They are named for the states in which they were isolated. All were initially isolated in chick embryos, and none had any passages in other animals. All other strains examined were isolated in this laboratory except strain Mid-Pac. which will be referred to later.

*Throat Washings.*—Specimens for virus isolation were obtained by having influenza patients gargle with 15 cc. of a 5 per cent normal horse serum in phosphate buffer. The washings were all taken during the acute stage of clinically typical epidemic influenza, usually while the patient was still febrile and within 4 days or less of the onset. Unless the washings were tested immediately they were stored on dry ice.

*Detection of Virus in Throat Washings.*—For attempts at virus isolation in chick embryos, throat washings were mixed with penicillin (165 units per cc.). For the amniotic method 0.3 cc. of washing was inoculated into the sacs of 13-day-old chick embryos by a modification of the method described by Burnet (1). The eggs were incubated for 4 days at 35°C., and the amniotic fluid was tested for chicken and guinea pig red cell agglutinins by the pattern method. Where repeated passages were performed by the amniotic route the lungs and tracheas of embryos were removed and ground in amniotic fluid for passage.

Virus isolations by the allantoic route were performed by the inoculation of 0.2 cc. of throat washing-penicillin mixtures into the sacs of 11-day-old chick embryos, followed by 2 or 3 days' incubation at 35°C. Tests for hemagglutinins were carried out by the pattern method.

Tests for virus were carried out in ferrets by inoculating these animals intranasally under light ether anesthesia with 1 cc. of untreated throat washing. Daily temperatures were taken, and sera obtained 2 weeks after inoculation were tested for antibody by the agglutination inhibition method (3).

*Strain Difference Tests.*—Cross inhibition tests for strain differences were carried out by the methods previously used (4). Antisera were obtained by infecting ferrets with a  $10^{-3}$  dilution of allantoic fluid, and sera were taken 2 weeks later. Simultaneous titrations of all immune sera against 4 agglutinating units of all the viruses were carried out by the agglutination inhibition tests using a photoelectric densitometer (3).

## EXPERIMENTAL

### *Titration of Influenza Virus in Throat Washings by Several Methods*

Although delicate methods of detecting influenza virus have been available for years, very little has been done to determine what concentrations of virus may be expected in the secretions of influenza patients. The frequent absence of detectable amounts of virus in throat washings of serologically proven cases of influenza has led to the assumption that the concentration is probably low. The development of new methods of virus detection in chick embryos provided a good means of investigating this problem.

The discovery by Rickard *et al.* (5) of the feasibility of inoculating the embryo by the allantoic route for primary virus isolation has led to the wide use of this method. The amniotic method of inoculation, which was developed earlier, has been used very little in the United States. While work at this laboratory has shown that with a group of throat washings the amniotic method gives a much greater number of positive isolations than the allantoic method (2), nothing so far has shown what the actual difference in sensitivity of virus detection might be.

Of forty-five throat washings from persons ill of influenza A in the 1943–44 epidemic, eight specimens yielded virus on inoculation into chick embryos by the allantoic route. These washings presumably had the highest virus titers and were investigated further. Two washings were found by amniotic titration to contain very high concentrations of virus, and these were tested by two other methods.

From one of these two washings (973), virus was obtained by amniotic inoculation even in a dilution of  $10^{-6}$  of the garglings (Table I). This result was confirmed by repetition and by subinoculation of the first-passage positive embryos. With the same washing, positive serological results were obtained with ferrets that had been inoculated with a throat-washing dilution of  $10^{-5}$ . But titration of washing 973 in chick embryos by the allantoic route gave positive results only through a  $10^{-2}$  dilution. A second throat washing (1004) was lower in titer but showed the same differences by the three methods of titration. Two other washings were titrated in the chick embryo amniotic sac with positive results in dilutions of  $10^{-3}$  and  $10^{-4}$ . It should be emphasized



that these high levels are exceptional and as a group made up less than 10 per cent of the total. The results confirm what was inferred from previous experience, that the amniotic method is definitely the most sensitive for the detection of virus. Ferret titration is a little less sensitive while, with the 1943 material, isolation by allantoic inoculation required of the order of 10,000 times more virus than isolation by the amniotic technique. It should be mentioned in connection with later comparisons with influenza B that these washings had been stored for 12 months on dry ice before being finally tested.

TABLE I  
*Comparative Titers of Virus in Throat Washings by Three Different Methods*

Dilution of throat washing	Throat washing 973 (1943-A)			Throat washing 1004 (1943-A)		
	Allantoic sac	Amniotic sac	Ferret-immune response	Allantoic sac	Amniotic sac	Ferret-immune response
10 <sup>0</sup>	6/6	4/5	1:256	4/4	—	—
10 <sup>-1</sup>	2/4	3/3	—	1/6	—	1:700
10 <sup>-2</sup>	2/4	3/3	—	0/6	—	1:1024
10 <sup>-3</sup>	0/6	5/5	1:475	—	4/4	1:350
10 <sup>-4</sup>	—	—	1:525	—	4/6	1:300
10 <sup>-5</sup>	—	—	1:300	—	1/5	1:32
10 <sup>-6</sup>	—	3/6	1:32	—	0/5	—
10 <sup>-7</sup>	—	0/4	—	—	—	—

In the chick embryo tests the numerator indicates the number of fluids showing positive chick cell agglutinins and the denominator the number of inoculated embryos that survived the incubation period. The titers given under the ferret test indicate the agglutination inhibition titer of serum taken 2 weeks after inoculation for 4 units of the PR8 strain. Inhibition titers of 1:32 are within *normal* inhibitory levels.

#### *Isolation of Virus by the Amniotic Route*

Of forty-five throat washings obtained in the influenza A epidemic in 1943-44, 74 per cent were found to contain virus when tested by the amniotic technique. However, in the influenza B epidemic of 1945-46, eighty-two throat washings were examined, of which only twenty-five (30 per cent) yielded virus. While it is conceivable that some of this difference may have been the result of variation in case sampling, this is unlikely since almost all of the later washings were from serologically positive cases. Other evidence makes it seem likely that the difference was due to the nature of the strains concerned.

In the influenza A epidemics it was unusual to obtain positive results from serial amniotic passage when the initial passage was negative, while in the influenza B epidemic 44 per cent of the strains isolated were first detected only on second passage, indicating that B virus took longer for detectable growth in the amniotic sac. A similar result was obtained by Burnet, Stone, and

Anderson (6), who state that with influenza B virus a 5-day incubation period gave better results than a 4-day one.

Attempts to isolate influenza B virus in dilutions of throat washings beyond  $10^0$  and all attempts to culture the virus initially in the allantoic sac completely failed.

### *Detection of Virus in Ferrets*

In influenza A epidemics the ferret has been successfully used for the detection of virus in numerous instances, but in our experience influenza B throat washings (1945-46) consistently failed to elicit any serological response in this animal. Eleven throat washings, proved by amniotic isolation to contain influenza B virus, gave completely negative results in the ferret. Only a suspension of lung tissue from a patient dying of hemorrhagic tracheobronchitis (strain Sinai 45) consistently infected these animals. However influenza B virus isolated in the egg and then administered to ferrets produced a good serological response and sometimes a febrile reaction; and the infection could be readily passed by the use of turbinate, but not lung, suspension.

### *Stability of Strains on Freezing and Storage*

In general, strains of influenza A virus are quite stable on prolonged storage at low temperature. In this laboratory influenza virus has been isolated from throat washings and stored for 5 years in cellulose nitrate tubes at  $-72^{\circ}\text{C}$ . The influenza B strains, on the other hand, are apparently very sensitive to freezing and storing at low temperatures. It was observed that if throat washings containing B virus were inoculated into eggs before freezing better results were obtained than if they were first frozen. Six washings were tested for virus before and after freezing, and the results recorded in Table II show that all washings became negative for virus after 4 months or less of storage at  $-72^{\circ}\text{C}$ . Tests were undertaken with egg-passage B virus stored under similar conditions and tested after several intervals. Table III shows the marked loss of titer with storage under these conditions. The same strain stored in sealed glass ampules did not lose titer over an even longer period, so that it seemed possible that the inactivation was due to diffusion of  $\text{CO}_2$  through the cellulose nitrate storage tubes. A comparison of the pH stability of this strain of B virus and the Lee strain showed no difference however. It seems reasonably clear that the B strains of 1945-46 were especially susceptible to deterioration with freezing and storage at low temperature.

From what has been presented so far in relation to the isolation of influenza B virus from throat washings, the conclusion would be justified that the difficulties encountered were due essentially to the low titer of virus in the garglings. However, in other respects these strains were so peculiar that it is

felt that the difficulty was more probably one of adaptation of the agent to various media.

TABLE II

*Isolation of Influenza B Virus before Freezing and after Freezing and Storage at Low Temperature*

Throat washing	Before freezing		Frozen and stored 1 wk.		Frozen and stored 4 mos.	
	1st passage	2nd passage	1st passage	2nd passage	1st passage	2nd passage
1	*3/3	—	†0/5	*1/5	†0/5	†0/4
2	*3/4	—	†0/4	*4/4	†0/3	†0/4
3	*3/4	—	†0/5	†0/5	—	—
4	*2/6	—	†0/5	†0/5	—	—
5	†0/6	*	—	—	†0/4	†0/3
6	†0/5	*5/5	—	—	†0/5	†0/3

\* Successful isolation.

† Negative isolation attempt.

—, no test.

Numerator = number of positive (hemagglutinin) embryos.

Denominator = total number of surviving embryos tested.

TABLE III

*Allantoic Titrations on a Strain of Egg Passage Influenza B Virus (Strain Sinai 45) before and after Storage at Low Temperature*

Dilution of allantoic fluid	Before freezing	Stored in lusteroid tubes at -72°C. for			Stored in sealed glass for 60 days
		3 days	7 days	14 days	
10 <sup>0</sup>				6/6	
10 <sup>-1</sup>				2/6	
10 <sup>-2</sup>		5/6	5/6	1/6	
10 <sup>-3</sup>		5/5	3/6	0/6	
10 <sup>-4</sup>	6/6	4/4	1/6	0/6	6/6
10 <sup>-5</sup>	4/5	2/4	0/6		4/4
10 <sup>-6</sup>	3/6	2/4			4/4
10 <sup>-7</sup>	2/6	2/4			1/4
10 <sup>-8</sup>	0/5				0/4

Numerators indicate the number of embryos with allantoic fluid yielding positive tests for hemagglutinin, and denominators the number of embryos inoculated that survived the incubation period.

#### *Adaptation of Viruses to the Allantoic Sac*

When influenza A strains, isolated in the amniotic sac, are subsequently titrated in both the allantoic and amniotic sac it is common for the two titrations to have similar end points, as shown in Table IV. In this laboratory

all A strains tested have adapted rapidly to the allantoic sac in the first passage, even the O form described by Burnet and Bull (7). The B strains of 1945, when detected in amniotic fluids were almost always present in high titer (1:500 or more), as tested by the pattern method. In spite of this, subculture of such fluids in the allantoic sac was difficult and adaptation was very slow. Titrations of successive allantoic passages of strain Sinai 45 gave ir-

TABLE IV

*Comparative Amniotic and Allantoic Titrations of an Influenza A and an Influenza B Virus Strain on Primary Isolation and after Passage in Chick Embryos by the Allantoic Route*

Dilution of inoculum	Strain 1004 (1943-A)				Strain Sinai (1945-B)							
	Titration of throat washing		Passage 1		Titration of lung suspension		Passage No.					
	Amniotic	Allantoic	Amniotic	Allantoic	Amniotic	Allantoic	1		6		10	
							Amniotic	Allantoic	Amniotic	Allantoic	Amniotic	Allantoic
10 <sup>0</sup>		4/4										
10 <sup>-1</sup>		1/6			2/3*	0/5				4/4		
10 <sup>-2</sup>		0/6			0/5			5/6		4/4		
10 <sup>-3</sup>	4/4							4/6		3/4		
10 <sup>-4</sup>	4/6							0/6		2/4		3/5
10 <sup>-5</sup>	1/6*		4/4	6/6				1/6		0/4	4/5	3/5
10 <sup>-6</sup>	0/5		4/5	3/6			2/5	0/6	3/4		5/5	0/6
10 <sup>-7</sup>			4/5	1/6			0/6		5/5		5/6	
10 <sup>-8</sup>			0/4	0/6					0/5		2/4	
Approximate amniotic allantoic titer ratio...	10,000		10		—		1000		1000		1000	

Strain 1004 was obtained from throat washings and strain Sinai from a human lung.

\* Indicates the amniotic fluids used for second passage, and titration; thereafter allantoic fluid was used for passage.

regular and spotty results, with unpredictable ups and downs, and a large number of passages was required to bring about full allantoic adaptation.

This was further shown by the simultaneous titration of different allantoic passages of strain Sinai 45 in both the amniotic and allantoic sacs (Table IV). With A strains, as previously noted, the amniotic/allantoic titer ratio of 10,000/1 on initial isolation rapidly changed to 10/1 on titration of first passage material, while with B strains the ratio persisted in the neighborhood of 1,000/1 for ten passages. One influenza B strain (Mid-Pac.) was not fully adapted to the allantoic sac after forty passages. Allantoic fluids yielded hemagglutinin titers (pattern method) of 1:8, while amniotic subinoculation at the same passage level yielded amniotic fluids with titers of 1:500 and over.

Attempts to adapt egg-passage strains of influenza A and B virus to mice are described in another paper (8). It is sufficient to point out here that A strains in general adapt readily, while the B strains of 1945-46 could not be maintained in mice without prior ferret passage. And even after such treatment the B strains did not increase much in virulence after many mouse passages.

### *Isolation of O Forms of Virus from Throat Washings*

Burnet and Bull have described what they refer to as the O (original) form of influenza virus, encountered on initial isolation in the chick embryo by the amniotic route (7). The O form is notable primarily for its high capacity for agglutinating guinea pig cells, with a much lower agglutinin titer for chicken cells. This type of virus disappears promptly on further passage in chick embryos unless special precautions are taken to preserve it. It is also characterized by good adaptation to growth in the amniotic sac and poor growth in the allantoic sac. Mice were found to be relatively insusceptible to infection with the O form of virus. Most of the work reported has been in connection with influenza A strains, but in a special study by Burnet *et al.* (9) the form was described in a strain of influenza B virus.

Many of the A strains isolated from the 1940-41 and 1943-44 epidemics showed O characteristics on initial isolation. Numerous attempts were made to maintain some of these strains in the O form by passage *via* the amniotic route in limiting dilutions. All of the techniques described by Burnet *et al.* (9) for maintenance of O strains were carefully followed. Each passage was carried out in a series of dilutions, lung and trachea were dissected out and tested for chick cell and guinea pig cell agglutinins, and only material showing a high guinea pig/chick cell titer ratio was passed. Before passage the chicken cell agglutinins were adsorbed out with chicken cells, and incubation was restricted to 2 or 3 days. In spite of all these precautions it was not found possible to maintain the O form through more than one or two passages without complete reversion to the D (derivative) form. Even in those instances in which the O form was briefly maintained it occurred in only a few embryos out of many and then not always in the dilutions near the end point. The results were further obscured by the tendency of O forms to agglutinate chicken cells in such a manner that the end points were long drawn out, with many consecutive dilutions showing a low grade of pattern formation. This made it difficult to assign reproducible chicken cell titers to a suspension.

Of the twenty-five influenza B strains isolated from the 1945-46 epidemic not one showed the slightest tendency toward the high guinea pig/chicken cell titer ratio on initial passage which is characteristic of the O form. In spite of the absence of this evidence of the O form, these strains had a uniformly high degree of adaptation to growth in the amniotic sac, poor adaptation to the allantoic cavity, and low infectivity for mice. These characteristics have all been described by Burnet and Bull (7) as corollaries of the O phenomenon,

and yet they exist in recently isolated B strains without the primary requisite of the O form, namely a high guinea pig/chicken cell titer ratio.<sup>1</sup>

It is frequently difficult to type freshly isolated strains of influenza virus because of the high degree to which their agglutination is inhibited by normal

TABLE V

*Summary of the Differences in Behavior of Influenza Virus Strains from Two Epidemics of Influenza A and One of Influenza B*

	Influenza A 1940-41 1943-44	Influenza B 1945-46
Per cent of throat washings yielding virus after amniotic inoculation	74	31
Per cent of washings yielding virus after allantoic inoculation	11	0
Ferret-immune response as indication of presence of virus in throat washing	Not quite as satisfactory as amniotic method	Gave no positive results, except with human lung material
Adaptation of virus to the amniotic sac	Very rapid	Very rapid
Value of repeated amniotic passage for initial isolation of virus	Little value, most strains take on first passage or not at all	44 per cent of strains isolated were first detectable only after two passages
Adaptation of virus to growth in allantoic sac	Very rapid, maximal adaptation on initial passage	Slow, poor, and difficult. Titrations irregular and titer often poor even after repeated allantoic passages
<i>In ovo</i> amniotic/allantoic titer ratios of passage strains	Usually 1/1 or 10/1	Often 1000/1 even after repeated allantoic passage
Mouse adaptation of egg passage virus	Easy, rapid. Maximum titer of virus occurs on initial passage	Difficult, and detectable virus disappears after one or two passages
O forms, guinea pig/chicken cell agglutinin titer ratio	O form fairly common on initial passage	No O forms seen
Stability at -72°C.	Very stable	Rapid loss of titer
Strain differences after embryo isolation	None between strains of same year and very slight between the two outbreaks	No strain differences found

serum. This finding was equally true of both influenza A and B virus. After repeated passage in the allantoic sac this high non-specific inhibition tends to disappear.

<sup>1</sup> Burnet *et al.* (6), investigating an epidemic of influenza B (1945) in Australia, also failed to find O forms.

Some of the differences in behavior of influenza A and B strains are summarized in Table V.

### *Study of Strain Differences by Agglutination Inhibition*

Ever since the original descriptions of differences among strains of influenza A virus (10-12) these antigenic variations have loomed as a potentially important factor in the epidemiology of influenza, in the natural resistance of populations to infections, and in resistance induced by vaccination.

Differences have been described as occurring among strains isolated in the same epidemic. In some instances the differences were great though they defied any very detailed classification into antigenic types. This early work was done with cross-protection tests in mice. More recently it has been demonstrated (4) that the antigenic differences among many of these early strains could be confirmed by cross-tests using the *in vitro* agglutination inhibition technique. After showing that the *in vitro* test was capable of demonstrating antigenic differences, it was found that strains isolated by the amniotic method from the same epidemic (1940-41) showed no detectable antigenic differences even when taken from widely separated points in the epidemic. On the other hand two strains isolated in ferrets and passed in mice did show definite antigenic differences from each other and from the embryo-isolated strains. This suggested that the strain differences first described in 1935 might be the result of ferret and mouse adaptation, a possibility which has actually been shown to occur (8). It therefore seemed profitable to study further the occurrence of strain differences by the *in vitro* technique.

The performance of cross-tests by the *in vitro* method is simple, and the results for each serum are expressed as a ratio of the homologous over the heterologous titer. An empiric correction is applied to the results. This has been fully discussed previously (4) and it is sufficient to emphasize that it serves mainly to render differences between strains reciprocal and to eliminate the paradox of sera which give higher than homologous titers with heterologous strains. The limit of error of the test is about one-half of a twofold difference on a logarithmic scale.

In the first cross-test performed six strains of influenza B were compared.

The strains include the Lee strain (13) isolated in 1940 in ferrets and mice and five strains isolated during 1945 from widely separated geographic areas. An influenza B epidemic occurred in the Territory of Hawaii during the months of June and July, 1945, during which time roughly 10 per cent of the population was affected. A strain (TW39) was isolated from a soldier on Oahu during June, 1945, by members of the virus section of the 18th General Medical Laboratory (14). It was isolated in chick embryos by the amniotic route and was carried through thirty-five passages in the allantoic sac before it was received at this laboratory. Even after this treatment the strain did not show consistently good growth on further allantoic passage. It is referred to here as strain Mid-Pac. Strains NH 1 and NH 2 were isolated from student military personnel stationed at Yale during December, 1945, also by the amniotic

route. Strain Cox was obtained from a patient at the New York Boys Vocational School, in November, 1945, and strain Sinai 45 was obtained from the lung of a patient who died of hemorrhagic tracheobronchitis at Mt. Sinai Hospital, New York City, also during November, 1945. All of the 1945 strains were passed from eight to forty times in the allantoic sac before use. The hemagglutinin titers of two strains (NH 2 and Mid-Pac.) were so low in allantoic fluid that they could not be used for the test, and it was necessary to grow the strains in the amniotic sac to obtain higher titer material.

The serum titers and the results of the cross-tests are shown in Fig. 1. These are very clear cut and show that none of the 1945 B strains studied differed

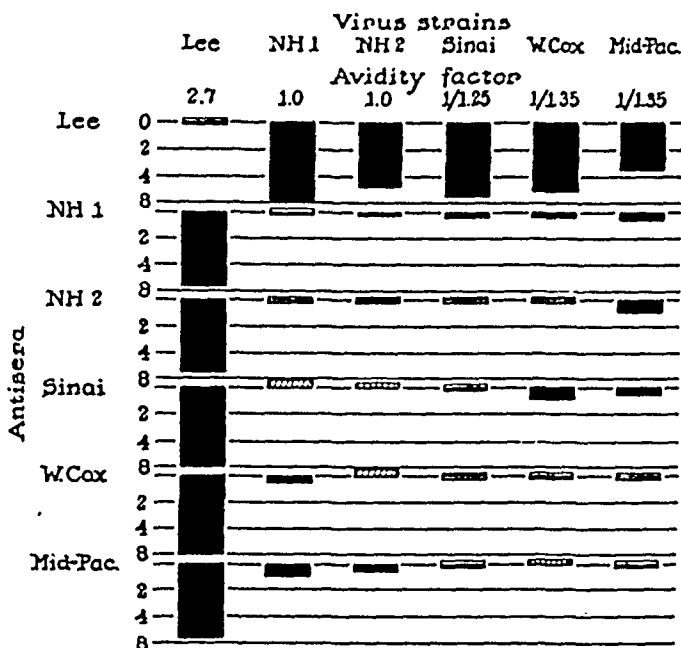


FIG. 1. Cross-tests between strains of influenza B virus. The results of agglutination inhibition tests between various strains of influenza B virus and their respective ferret antisera are expressed for a given serum in terms of the ratio of homologous over heterologous titers. The height of the black bars indicates the degree of difference between strains.

Corrections for avidity factor are included at the top of the figure. This renders the strain differences more nearly reciprocal and may be made necessary by varying degrees of aggregation in virus suspensions. All of the strains were isolated in 1945 except Lee, which was obtained in 1940.

significantly from any of the others, not even the Mid-Pac. strain, which came from a case occurring 6 months earlier than the others. All the 1945 strains show distinct, and within the error of the method, equal differences from the mouse-adapted Lee strain. The corrections applied were small and affected mainly the distribution of differences with the Lee strain. This test with influenza B virus confirms the result previously obtained with influenza A strains of 1940-41 in that there appears to be very little strain variation evident in virus from widely separated sources.



In Fig. 2 are shown the results of a similar test with strains from four different states, obtained during the influenza A epidemic of 1943-44. While

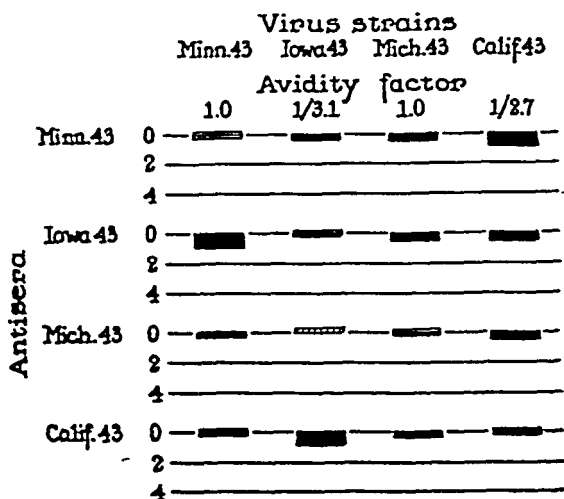


FIG. 2. Cross-tests between influenza A strains of 1943-44. The strains tested were all obtained from the influenza epidemic of 1943-44 and were isolated initially in chick embryos.

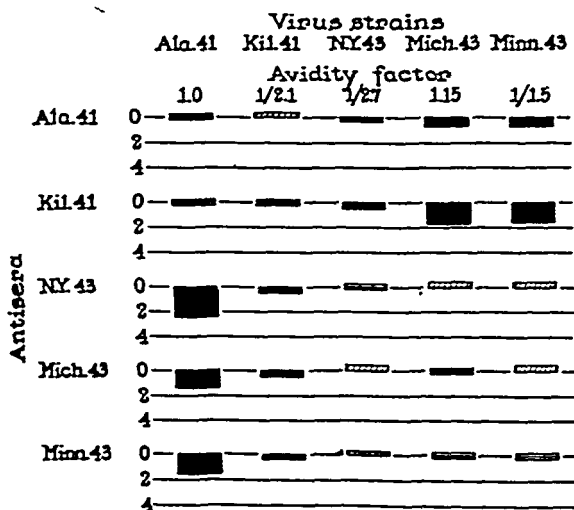


FIG. 3. Cross-tests of influenza virus strains from two epidemics. A comparison of strains of influenza A virus isolated in the epidemics of 1940-41 and 1943-44.

the corrections are large, no essential or significant differences were found between strains obtained from these four areas. All were egg-isolated strains, primarily obtained in the allantoic sac.

A cross-test was also performed between A strains of 1940-41 and of 1943-44, the latter including two strains shown in Fig. 2 and an additional one from New York (Fig. 3). The 1940-41 strains were both obtained from different places in Alabama. The similarity of strains isolated in the same year is striking, while there seems to be some slight change in antigenic pattern between the 2 years. This latter antigenic difference is small and in many crosses it is not significant in degree, but it is so consistently present in all the 1940-41 vs. 1943-44 cross-tests that it probably represents a small but real change.

#### DISCUSSION

The work here described further underlines the advantages of chick embryos over ferrets for isolation of influenza virus even from the standpoint of sensitivity in virus detection. More marked, however, is the demonstration of the advantage of inoculation by the amniotic route over allantoic inoculation, especially in the case of influenza B virus, which in this laboratory was successfully isolated only by the amniotic route. Success of virus isolation is heightened when washings are directly inoculated into eggs shortly after they are obtained. If freezing is necessary, it seems better that washings be stored in glass ampules rather than in lusteroid tubes. The difficulty of maintaining some B strains in the allantoic sac is evident, and material for serological work can sometimes be more advantageously obtained by amniotic passage.

From the epidemiological standpoint the occasional occurrence of influenza patients with exceptionally high virus titers in their throat secretions is of interest. While the exact dilution of the throat secretions in the gargling mixture is unknown, a tenfold dilution would be a rough estimate. This would place the minimum number of infectious particles in throat secretions in one instance in the neighborhood of  $10^{7.5}$  per cc. It may well be that individuals with such concentrations of virus may be the ones mainly responsible for disseminating the disease. It is also possible that the occurrence of such high levels is more common than is indicated and that the peak level is maintained for only a short time.

The O phenomenon described by Burnet and Bull (7) has been confirmed in regard to its occurrence on initial isolation, but it has not been found possible to maintain this form on passage. Strains may differ in their tendency to shift to the D form, and it may be that those with a strong tendency to do so cannot be maintained in the O state. At present the importance of the O phenomenon seems somewhat obscure. The O-D shift apparently does not represent a loss of any potentialities but mainly a gain in chicken cell hemagglutinins. The difficulty of maintaining the virus in the O form hampers detailed study very greatly.

At the risk of some repetition it might be well here to restate the present position in regard to strain differences within the influenza A and B groups:

(1) Antigenic differences have been found between strains of the A, B, and swine types of influenza virus; in some instances the differences have been trivial but in others large, even between strains occurring in the same epidemic. (2) In every instance in which differences have been described they have occurred in strains which have been adapted to mice. (The cross-tests have been carried out by cross-neutralization in mice.) (3) These differences have been confirmed by cross-tests by the agglutination inhibition method, showing this test to be capable of detecting small changes in antigenic pattern. (4) With the use of this *in vitro* technique of comparison it has been shown that during the process of mouse adaptation the antigenic pattern shifts; and the shift in structure is not always in the same direction, so that two similar strains may become quite different after passage in mice. This observation alone would make the significance of the initial observations on differences questionable as regards their meaning for human strains. (5) Adaptation of human virus to the egg is apparently a less drastic procedure than mouse adaptation, and when egg-adapted viruses have been used for comparison of strains from epidemics, no significant antigenic differences have been revealed. This was the case in the influenza A epidemics of 1940-41 and 1943-44 and in the influenza B epidemic of 1945-46. Even a comparison between the 1940-41 and 1943-44 strains revealed only slight differences.

These observations uniformly point to the fact that strain differences in a given epidemic are negligible. At first glance this would seem to be unlikely when one realizes the enormous number of human passages some of these similar strains must have been subjected to, and especially in view of their antigenic lability with relatively few mouse passages (9). If this view of antigenic similarity is accepted it must mean that both the A and B influenza viruses have achieved considerable antigenic stability. An analogous finding was described by Magill and Francis, who observed little difference between the thirtieth and the two hundred eighty-fifth passage of the PR8 strain of influenza A virus in mice, indicating that antigenic stability was achieved before the thirtieth passage.

These findings also have a practical bearing on influenza prophylaxis by subcutaneous vaccination. The current preparation of vaccine authorized for distribution, and widely tested in the field, contains only mouse-adapted strains of virus. Among these strains is PR8, which has been most widely studied and differs from the egg-adapted A strains by a two- to fourfold margin. The second A strain in the vaccine is Weiss, on which no antigenic studies have been published; and finally there is the Lee strain of influenza B virus, which differs considerably from the 1945-46 type. It would seem logical, in preparing vaccines, to substitute for these strains others which are as similar as possible to strains ordinarily current during epidemics in man. Experience in this laboratory suggests that egg-adapted strains would be a more logical

choice, providing they proved to be satisfactory in other ways, such as yielding high titer in eggs and good antigenic response in man. From the wide selection of such strains now available it should not be difficult to obtain a number which have the desired characteristics.

#### SUMMARY

Some of the peculiarities of strains of influenza A and B virus from two epidemics have been described. The influenza B virus of 1945-46, when compared with influenza A virus, proved to be much more difficult to isolate from human sources by any known means. Its adaptation to the chick embryo (by any route) or to mice was much slower than that of A virus. It did not keep nearly as well on storage at  $-72^{\circ}\text{C}$ . either in throat garglings or as passage material. Its adaptation to amniotic growth was usually much better than to allantoic growth even after repeated allantoic passages. It failed to show primary evidence of occurring in the O form, although many of the secondary O characteristics were present and persisted. Its titer in throat washings was not demonstrably high as compared with certain strains of A virus, which were demonstrated in garglings at dilutions of  $10^{-5}$  and  $10^{-6}$ .

The antigenic patterns of influenza A strains from two epidemics were compared. No antigenic differences of significant degree were found among the strains of either epidemic and the difference between the strains of the two epidemics was very slight. A similar study was made of the influenza B strains of the epidemic of 1945-46. This also showed complete lack of significant strain differences. The implications of these findings for influenza prophylaxis are discussed.

*Addendum.*—More recent experience with the influenza strains isolated from the A epidemic of early 1947 in the United States indicates that these strains show considerable antigenic differences from examples of the earlier outbreaks. The 1947 strains also differ from the earlier ones in that they are much more difficult to adapt to the allantoic sac and are readily neutralized in *in ovo* tests by normal ferret and normal rabbit sera. The occurrence of strain differences within the epidemic is being investigated.

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# THE EFFECT OF SULFHYDRYL GROUPS ON PNEUMONIA VIRUS OF MICE (PVM)

By MOGENS VOLKERT, M.D., AND FRANK L. HORSFALL, JR., M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

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The results of previous studies (1, 2) have shown that pneumonia virus of mice (PVM) is very unstable as regards the property of infectiousness but is remarkably stable with respect to its capacity to cause hemagglutination. In the course of studies described in the accompanying paper (3), on the virus-combining component present in the lungs of animal species susceptible to infection with PVM, evidence was obtained which indicated that the titer of the virus, as measured by hemagglutination, is influenced strikingly by the concentration of the lung tissue suspension containing it. Because the virus appeared to be affected deleteriously in suspensions of high concentration, an explanation for this unexpected finding was sought.

It is the purpose of this paper to present the results of experiments concerning the effect of the concentration of lung tissue suspensions on PVM. It will be shown that the virus is adversely affected in concentrated suspensions as well as in the presence of glutathione; that the effect is prevented in both instances by the addition of iodoacetamide; and that, in all probability, the decreased stability of the virus in concentrated lung suspensions or in the presence of glutathione is caused by the presence of sulfhydryl groups.

## Methods

*Virus.*—Pneumonia virus of mice (PVM), strain 15 (4), was employed. Suspensions of infected mouse lungs in saline were prepared as described in the accompanying paper (3). Such suspensions have been shown to contain combined infectious virus (2). Heat-released virus was obtained by heating suspensions at 70°C. for 30 minutes as described previously (2). Free infectious virus was obtained from intact infected lungs by the centrifugation technique described previously (3, 5). Infectivity tests in mice were carried out exactly as in earlier studies (1).

*Hemagglutination Tests.*—The technique of hemagglutination tests with mouse RBC and the method of estimating end points were identical with those previously employed (2).

*Glutathione.*—The glutathione used was obtained from a commercial laboratory.<sup>1</sup> A fresh solution adjusted to pH 7.0 in saline was prepared for each experiment and used promptly.

*Iodoacetamide.*—Dr. Rollin D. Hotchkiss of the Rockefeller Institute prepared and kindly supplied the iodoacetamide used. Solutions were prepared in saline.

## EXPERIMENTAL

*Stability of PVM in Mouse Lung Suspensions.*—Experiments were carried out to determine the effect of the concentration of mouse lung suspensions

<sup>1</sup> Schwarz Laboratories, Inc., New York.

on the PVM contained in them as evidenced by the capacity of the virus to induce infection and to cause hemagglutination following appropriate heating (2).

Suspensions of mouse lungs infected with PVM were prepared in saline. The concentration of the suspensions, in terms of the wet weight of the lungs, ranged from 50 to 10 per cent. Suspensions were held at various temperatures and at intervals aliquots were removed. Hemagglutination titers were determined after aliquots had been heated at 70°C. for 30 minutes. Infectivity titers were determined on unheated aliquots in mice.

The results of typical experiments are shown in Table I. It will be seen that, when suspensions were heated a short time after preparation, the hemagglutination titers of concentrated suspensions were not proportionately higher

TABLE I

*The Effect of the Concentration of Mouse Lung Suspensions on the PVM Contained in Them*

PVM mouse lung suspension	Suspension held		Infectivity titer M. S. 30	Hemagglutination titer following 70°C. for 30 min.
	Temperature	Time		
<i>per cent</i>	<i>°C.</i>	<i>hrs.</i>	<i>log</i>	
10	24	1	-4.21	512*
25	24	1	-4.14	256
50	24	1	—	256
10	4	24	-4.21	768
25	4	24	-4.00	0
10	37	10	—	512
50	37	10	—	32

\* Reciprocal of titer.

but actually were lower than those of 10 per cent suspensions. With increasing time even more strikingly discrepant results were obtained with concentrated suspensions. The marked decrease in hemagglutination titer which occurred with concentrated lung suspensions varied somewhat from one experiment to another, but it was found regularly that, after 10 hours at 37°C. or 24 hours at 4°C., only very little of the virus originally present in concentrated suspensions could be demonstrated by the hemagglutination technique.

It should be noted, however, that the capacity to induce infection was undiminished in concentrated lung suspensions which had been held at 4°C. for 24 hours, despite the fact that no virus was demonstrable in the same suspensions by the hemagglutination technique after appropriate heating. It appeared, therefore, that PVM was not actually destroyed in concentrated lung suspensions held at 4°C. even though it was rendered no longer demonstrable by the hemagglutination procedure.



In other experiments a fresh 50 per cent suspension of normal mouse lungs in saline was added to a 10 per cent suspension of infected lungs. Aliquots of the mixture were held at 37°C. or 4°C. and at intervals samples were removed and tested as before. The results obtained were similar to those described above. The addition of normal lung tissue to suspensions of infected lungs caused a prompt reduction in the amount of virus which could be released by heating and, with increasing time, the amount of virus demonstrable by the hemagglutination technique progressively decreased.

*Effect of Glutathione on Stability of PVM.*—Because tissue suspensions provide reducing conditions, experiments were carried out to determine whether the addition of a reducing substance, e.g. glutathione, to infected mouse lung suspensions would exert an effect upon PVM similar to that observed with concentrated suspensions.

To 10 per cent suspensions of mouse lungs infected with PVM (combined infectious virus<sup>2</sup>) a fresh solution of glutathione was added to give a concentration of 0.005 M. The mixtures were held at 24°C. and at intervals samples were removed. Infectivity titers were determined in the usual manner in mice. Hemagglutination titers were determined following heating at 70°C. for 30 minutes.

The results of representative experiments are shown in Table II. It was found that the addition of 0.005 M glutathione to PVM mouse lung suspensions had no effect upon the infectivity titer of the suspensions but, following heating of the mixtures, no virus could be demonstrated by the hemagglutination technique. These results indicate that in the presence of glutathione PVM is incapable of withstanding the degree of heating employed as routine to release the virus from combination with the lung tissue component (2, 3).

If the effect of glutathione on the heat stability of PVM were due to the sulfhydryl groups of the compound, it would be expected that the addition of iodoacetamide, which combines with sulfhydryl groups (6), would prevent the effect of glutathione on the virus.

To 10 per cent suspensions of infected mouse lungs was added either iodoacetamide solution to give a concentration of 0.01 M or a fresh solution of glutathione to give a concentration of 0.005 M. The mixtures were held at 24°C. and at intervals samples were removed. To aliquots of those mixtures which contained glutathione, iodoacetamide was then added. Hemagglutination titers were determined following heating in the usual manner.

The results of typical experiments are shown in Table II. It is evident that the addition of iodoacetamide to a lung suspension containing glutathione completely prevented the effect of glutathione on the heat stability of PVM. Moreover, when 0.005 M glutathione was employed, iodoacetamide (0.01 M)

<sup>2</sup> As indicated in the accompanying paper, combined infectious virus refers to PVM present in ground lung tissue suspensions; although fully infectious the virus does not cause hemagglutination because it is in combination with a lung tissue component.

could be added 1 hour after the addition of glutathione and elimination of the effect of glutathione on the virus was obtained.

Similar experiments were carried out with free PVM. Both heat-released (2) and free infectious virus (5) were employed. It will be recalled that free PVM possesses the advantage that it causes hemagglutination directly and, unlike combined PVM, does not require further treatment to unmask this property.

TABLE II  
*The Effect of Low Concentrations of Glutathione and Iodoacetamide on PVM*

PVM	Added before incubation		Incubation at 24°C. for	Added after incubation		Infectivity titer M. S. 50	Heating	Hemagglutination titer
	Gluta- thione	Iodoacet- amide		Iodoacet- amide				
Combined infec- tious virus	0	0	1 hr.	0	<i>log</i> -4.21	70 C. for 30 min.	512*	
“ “	0.005 M	0	1 “	0	-4.11	“ “	0	
“ “	0	0.01 M	1 “	0	—	“ “	512	
“ “	0.005 M	0	1 “	0.01 M	—	“ “	512	
Heat-released virus	0	0	2 hrs.	0	—	None	512	
“ “	0.005 M	0	2 “	0	—	“	512	
“ “	0.005 M	0	1 min.	0	—	70 C. for 30 min.	0	
“ “	0.005 M	0	2 hrs.	0	—	“ “	0	
“ “	0.005 M	0	24 “	0	—	“ “	256	
“ “	0	0.01 M	1 hr.	0	—	“ “	512	
“ “	0.005 M	0	1 “	0.01 M	—	“ “	512	
“ “	0.005 M	0.01 M	1 “	0	—	“ “	512	
Free infectious	0.005 M	0	1 “	0	—	None	512	

\* Reciprocal of titer.

The results of these experiments also are presented in Table II. It will be seen that the presence of glutathione (0.005 M) caused no reduction in the hemagglutination titer of either heat-released or free infectious virus, but that, when the mixtures were heated within 2 hours of their preparation, the capacity to cause hemagglutination was lost. It was found that the effect of glutathione on the virus was produced promptly; 1 minute after the addition of glutathione no virus could be demonstrated by means of hemagglutination if the mixture was heated at 70°C. With increasing time the effect of glutathione progressively decreased and when mixtures were heated 6 to 24 hours after preparation maximal hemagglutination titers were again obtained. It is well known that

glutathione is unstable in solution and that on standing it gradually becomes oxidized to the disulfide form. It appears probable that elimination of the effect of glutathione on PVM with increasing time of incubation of a mixture is similar to the effect which was obtained when iodoacetamide (0.01  $\mu$ ) was added 1 hour after the mixture had been prepared.

It was of interest to determine whether concentrations of glutathione greater than 0.005  $\mu$  would cause even more marked effects on PVM. Therefore, further experiments, similar to those described above, were performed.

To preparations of combined infectious virus (10 per cent suspensions of infected mouse lungs), heat-released virus, or free infectious virus was added a fresh solution of glutathione to give a concentration of 0.05  $\mu$ . The mixtures were held at 37°C. and at intervals samples were removed and their hemagglutination titers determined. To aliquots of these mixtures, either before incubation or following it, a solution of iodoacetamide was added to give a concentration of 0.1  $\mu$ .

The results of typical experiments are presented in Table III. It was found that, when 0.05  $\mu$  glutathione was present in suspensions containing combined PVM, it was necessary to add iodoacetamide (0.01  $\mu$ ) immediately if the effect of glutathione on the virus was to be inhibited. One hour after the addition of glutathione in this concentration, the addition of iodoacetamide failed to eliminate the effect of glutathione on the hemagglutinating capacity of the virus.

Moreover, with 0.05  $\mu$  glutathione, striking direct effects were obtained on the hemagglutination titer of free PVM. As is shown in Table III, the hemagglutination titer of either heat-released or free infectious virus rapidly decreased in the presence of 0.05  $\mu$  glutathione and, after 1 hour at 37°C., little or no virus was demonstrable by the hemagglutination technique even when heating was not employed. With free infectious virus it was found that the capacity of the virus to induce infection also decreased rapidly in the presence of 0.05  $\mu$  glutathione. When iodoacetamide (0.1  $\mu$ ) was added simultaneously with glutathione, the striking reduction in hemagglutination titer was completely prevented but, if iodoacetamide was not added until 1 hour after the addition of glutathione, the reduction in hemagglutination titer was not reversed. Combined infectious virus, heat-released virus, and free infectious virus appeared to be affected in an identical manner by glutathione.

The results of these experiments indicate clearly that in the presence of glutathione the stability of PVM, either in the combined or the free state, is decreased. In the presence of relatively low concentrations of glutathione (*i.e.*, 0.005  $\mu$ ) the effect can be either prevented or eliminated by the addition of iodoacetamide. However, in the presence of relatively high concentrations of glutathione (*i.e.*, 0.05  $\mu$ ) the effect can be prevented but cannot be eliminated by the addition of iodoacetamide. The capacity of free PVM to cause hemag-

glutination is unaffected by low concentrations of glutathione but rapidly decreases, as also does the capacity to induce infection, when high concentrations are present. The reduction in the hemagglutination titer of free virus under these conditions can be prevented but not completely reversed by the addition of iodoacetamide.

TABLE III  
*The Effect of High Concentrations of Glutathione and Iodoacetamide on PVM*

PVM	Added before incubation		Incubation at 24°C. for	Added after incubation	Infectivity titer M. S. 50	Heating	Hemagglutination titer
	Glutathione	Iodoacetamide		Iodoacetamide			
Combined infectious virus	0	0	1 hr.	0	—	70 C. for 30 min.	512*
“ “	0.05 M	0	1 “	0	—	“ “	0
“ “	0	0.1 M	1 “	0	—	“ “	256
“ “	0.05 M	0	1 “	0.1 M	—	“ “	8
“ “	0.05 M	0.1 M	1 “	0	—	“ “	256
Heat-released virus	0	0	1 hr.	0	—	None	512
“ “	0.05 M	0	1 min.	0	—	“	256
“ “	0.05 M	0	30 “	0	—	“	32
“ “	0.05 M	0	1 hr.	0	—	“	0
“ “	0	0.1 M	1 “	0	—	“	256
“ “	0.05 M	0	1 “	0.1 M	—	“	16
“ “	0.05 M	0.1 M	1 “	0	—	“	256
Free infectious virus	0	0	1 “	0	—2.61	“	256
“ “	0.05 M	0	1 “	0	—0.72	“	2

\* Reciprocal of titer.

*Effect of Iodoacetamide on Stability of PVM in Lung Suspensions.*—The effect of glutathione on PVM appeared to be analogous to the effect of concentrated lung suspensions on the virus. Because iodoacetamide was capable of preventing the effect of glutathione and of eliminating its action under appropriate conditions, experiments were carried out to determine whether the addition of iodoacetamide to concentrated PVM lung suspensions would prevent the marked reduction in hemagglutination titer which occurs in such suspensions.

Suspensions of mouse lungs infected with PVM, which in terms of wet weight ranged from 50 to 10 per cent, were prepared in saline. To aliquots of these suspensions a solution of

iodoacetamide was added to give a concentration of 0.01 M. The suspensions and mixtures were held at 37°C. and at intervals samples were removed. To aliquots of the suspensions iodoacetamide (from 0.01 to 0.1 M) was then added, following which all samples were heated at 70°C. for 30 minutes and their hemagglutination titers determined in the usual manner.

The results of typical experiments are shown in Table IV. It will be seen that the addition of iodoacetamide did not alter the hemagglutination titer of 10 per cent suspensions and, moreover, that such suspensions could be held at 37°C. for 20 hours without the addition of iodoacetamide and still show maximal

TABLE IV

*The Effect of Concentration of Mouse Lung Suspensions and of Iodoacetamide on PVM*

PVM mouse lung suspension	Added before incubation	Incubation at 37°C. for	Added after incubation	Hemagglutination titer following 70°C. for 30 min.
	Iodoacetamide		Iodoacetamide	
<i>per cent</i>		<i>hrs.</i>		
10	0	0	0	512*
"	0.01 M	0	0	512
"	0	20	0	512
50	0	0	0	256
"	0	0	0.01 M	2048
"	0	10	0	32
"	0	10	0.01 M	1024
"	0	20	0	0
"	0	20	0.01 M	16
"	0	20	0.1 M	16
"	0.01 M	20	0	1024

\* Reciprocal of titer.

hemagglutination titers after appropriate heating. On the other hand, the addition of iodoacetamide to 50 per cent suspensions of infected lungs resulted in a marked increase in their hemagglutination titers following heating. In the presence of iodoacetamide the titer of 50 per cent suspensions was found regularly to be five times higher than that obtained with 10 per cent suspensions.

When iodoacetamide was added to 50 per cent suspensions and the mixture then was incubated for 20 hours, no significant decrease in hemagglutination titer occurred. Even when it was added following 10 hours' incubation of 50 per cent suspensions, an almost undiminished hemagglutination titer was obtained following heating, whereas during an identical period in the absence of iodoacetamide a very marked reduction in titer occurred. When the incubation period with such concentrated suspensions was 20 hours, in the absence of iodoacetamide no virus could be demonstrated by the hemagglutination technique.

The results of these experiments indicate clearly that iodoacetamide is capable of preventing the deleterious effect of concentrated lung suspensions upon the heat stability of PVM and even of restoring this. Moreover, they show that during incubation at 37°C. the virus in concentrated lung suspensions becomes progressively less stable to heat. After 10 hours' incubation this effect is almost completely eliminated by the addition of iodoacetamide but after 20 hours' incubation the effect is almost entirely irreversible even though much iodoacetamide is added.

#### DISCUSSION

The results obtained in this study indicate that the presence of sulfhydryl groups is an important factor which influences the stability of PVM. In the presence of sulfhydryl groups provided by 0.005 M glutathione the capacity of the virus to induce infection is unaltered but it is promptly rendered less stable to heat. The effect of this concentration of glutathione on the heat stability of the virus is eliminated either by the spontaneous oxidation of glutathione which occurs in solution or by the addition of iodoacetamide which combines with sulfhydryl groups. In the presence of sulfhydryl groups provided by 0.05 M glutathione irreversible changes in the virus take place, the capacity to induce infection is lost rapidly, and there is associated with this change a corresponding decrease in hemagglutination titer. Both free and combined virus are similarly affected by high concentrations of glutathione and, although the effect can be prevented completely by the addition of iodoacetamide, it is not eliminated by the addition of this substance, once it has occurred.

In concentrated lung tissue suspensions changes are induced in the virus which are strikingly similar to those which occur in the presence of glutathione. Under appropriate conditions (*e.g.*, 4°C.) in concentrated suspensions the capacity of the virus to induce infection is unaltered but marked instability to heat develops. This latter change, like that which occurs in the presence of glutathione, can be prevented by the addition of iodoacetamide. Similarly, if the reaction has not been allowed to proceed for too long a period (*e.g.*, 10 hours), the decreased heat stability of the virus can be eliminated by the addition of iodoacetamide. After a longer time an irreversible change in heat stability occurs analogous to that obtained with 0.05 M glutathione, and this is not eliminated by iodoacetamide.

The finding that the infectious as well as the hemagglutinating properties of PVM are both similarly affected in either concentrated lung tissue suspensions or in the presence of glutathione, and that these effects are prevented or eliminated in like degree by iodoacetamide, indicates that in both instances the alterations in the properties of the virus are dependent upon the presence of sulfhydryl groups.

## SUMMARY

Evidence is presented which indicates that PVM is affected adversely in concentrated lung tissue suspensions or in the presence of glutathione. Because iodoacetamide inhibits or eliminates these effects in a similar manner, it is concluded that sulphydryl groups are essential to their development.

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# STUDIES ON A LUNG TISSUE COMPONENT WHICH COMBINES WITH PNEUMONIA VIRUS OF MICE (PVM)

BY MOGENS VOLKERT, M.D., AND FRANK L. HORSFALL, JR., M.D.

*(From the Hospital of The Rockefeller Institute for Medical Research)*

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Pneumonia virus of mice (PVM) (1), like at least nine other animal viruses, possesses the capacity to cause agglutination of erythrocytes obtained from certain mammalian species (2-4). Unlike other viruses which cause hemagglutination however, PVM in suspensions of infected tissues requires treatment by special procedures in order that hemagglutination may be demonstrable. The results of detailed investigations (4) with PVM indicate that the virus particles themselves are responsible for hemagglutination and that the hemagglutination technique provides a means of measuring *in vitro* the concentration of either the virus or of antibodies against it. It was shown previously (4-6) that PVM may occur in at least two different physical states; in one, the virus particles are in firm combination with a lung tissue component; in the other, the virus particles are free, that is, uncombined. When in combination with lung tissue particles the virus is incapable of combining with erythrocytes. Hemagglutination with free virus is but one evidence of the combination which occurs between the virus and the tissue component present in suitable erythrocytes. In earlier papers (4, 6) it was suggested that the capacity of lung tissue to combine with the virus might be an important factor in the pathogenesis of infection with the agent. Moreover, the possibility was entertained that the virus is able to infect susceptible cells only when it is in the free state.

It is the purpose of this paper to present the results of further experiments with PVM and with the lung tissue component which combines with the virus. A technique has been devised by means of which the virus-binding capacity of tissue suspensions can be measured with some precision. It will be shown that there is a close correlation between the degree of susceptibility to infection with PVM and the amount of the virus-binding component present in the lung tissues of various animal species. Evidence will be presented which indicates that free virus is taken up readily and bound by the cells of the intact normal lung, whereas with combined virus such fixation does not occur until the virus-tissue component complex has been split. It will be shown that enzymes are capable of acting on the tissue component and of releasing free virus from combination with it. Reasons will be given for thinking that enzymatic splitting of the complex probably is an initial step essential to the development of infection when combined virus is given.

### Methods

*Virus.*—Pneumonia virus of mice (PVM), strain 15 (1), was used exclusively. It was maintained by occasional passage in albino Swiss mice and stored as a 10 per cent suspension of infected mouse lungs at  $-70^{\circ}\text{C}$ . Preparations of combined virus or of free virus were obtained as described in previous communications (4, 6). Unless otherwise indicated, combined virus was that present in ground suspensions of infected mouse lungs, whereas free virus was that present in similar suspensions which had been heated at  $70^{\circ}\text{C}$ . for 30 minutes.

*Infectivity Tests.*—Titrations of virus infectivity employing serial tenfold dilutions were carried out in albino Swiss mice according to the technique described in detail previously (7). End points were calculated by the 50 per cent maximum score method as in previous studies (7).

*Hemagglutination Tests.*—The titrations of hemagglutinating activity were carried out with serial twofold dilutions of virus suspensions and 0.8 per cent mouse RBC. The technique and the method of estimating end points were identical with those previously described (4).

*Tissue Suspensions.*—Suspensions of appropriate animal tissues in saline were made by mixing in the Waring blender for 2 minutes. Suspensions were used on the day they were prepared or were stored at  $-70^{\circ}\text{C}$ .

### EXPERIMENTAL

*Virus-Combining Capacity of Normal Tissues.*—Before it was possible to obtain quantitative information concerning combination between PVM and various tissues, it was necessary to devise a method by means of which the virus-binding capacity of a tissue suspension could be measured. Curnen and Horsfall (4) showed that both time and temperature are important factors in the reaction between PVM and tissue particles. These observations were readily confirmed, and it was found that when appropriate proportions of virus and lung tissue were used all of the virus was bound by the tissue in a period of 30 minutes at  $37^{\circ}\text{C}$ . If larger amounts of virus were used than the tissue could combine with readily in 30 minutes, additional binding of the virus by the tissue occurred over a period of hours. Even in the presence of excess virus, however, 90 to 95 per cent of the virus which eventually the tissue could bind was combined within the first 30 minutes of the reaction. If larger amounts of tissue were used, the time required to bind all the virus was proportionately less than 30 minutes.

In the light of these findings, the following technique was employed to determine the virus-combining capacity of a tissue suspension: 10 per cent tissue suspensions were prepared in saline and were mixed in the Waring blender for 2 minutes. The technique of preparation of tissue suspensions influences the virus-binding capacity; inadequate mixing or grinding results in a lower virus-binding titer as also does centrifugation. To obtain comparable results, therefore, all tissue suspensions were prepared under uniform conditions and were not centrifuged. Serial twofold dilutions of tissue suspensions were made in saline and to each tube was added 8 hemagglutinating units of virus. The mixtures were kept at  $37^{\circ}\text{C}$ . for 30 minutes and then centrifuged at 12,000 R.P.M. for 10 minutes. From each tube 0.2 cc. of the supernate was taken and to it was added an equal volume of an 0.8 per cent mouse RBC suspension. The presence or absence of hemagglutination was determined after 1.5 hours at room temperature. The last tube in which no hemagglutination occurred was taken as representing the

end point and it indicated the smallest amount of the tissue suspension which was able to bind completely 8 units of virus. This quantity was taken as the virus-combining titer of the tissue suspension.

It should be pointed out that two other factors, in addition to the tissue component itself, may complicate the interpretation of the results of tests for binding capacity with PVM. It will be recalled that the red blood cells of both mice and hamsters are able to combine with large amounts of PVM (4). Also, the blood of various mammalian species may contain antibodies against the virus which inhibit hemagglutination (4, 8). In hamsters and mice; therefore, a reliable result is obtained only when organs which have been freed of blood are employed. Perfusion of organs with saline to remove blood is most easily accomplished in the case of the lungs, kidneys, and brain, and consequently suspensions of these organs were tested. In addition to hamsters and mice, cotton rats are also susceptible to experimental infection with PVM. However, cotton rat red blood cells are not able to combine with the virus (4) but antiviral antibodies could be responsible for erroneous results. If cotton rats which possess no antibodies against PVM are selected, all of their organs can be tested for the presence of virus-binding component.

Tests for the presence of substances which combine with PVM were carried out with suspensions of various normal tissues obtained from several animal species. The results are shown in Table I. It is evident that among the various organ suspensions tested only mammalian lung suspensions were capable of binding the virus. It should be pointed out that the virus-binding capacity of the lungs from different animal species closely paralleled the degree of susceptibility of these species to infection with PVM. Hamsters and mice are most susceptible to infection with the virus (7, 8) and their lungs show the highest virus-combining titer. Cotton rats are less susceptible to infection with the virus (8) and their lungs show a virus-combining titer eightfold lower than that of mouse or hamster lungs. Rabbits and guinea pigs have not been proven to be susceptible to experimentally induced infection with PVM, but serological evidence (8) indicates that both species, and also human beings, develop inapparent infections naturally with the virus. With lung tissue from each of these three species the capacity to bind PVM was present but the combining titer was very low. As is shown below, both chick embryos and duck embryos appear to be completely insusceptible to infection with the virus. It appears of considerable interest that with avian embryo suspensions it was not possible to demonstrate the presence of any virus-binding capacity whatsoever.

Attempts were made to obtain the virus-binding component from intact lungs without subjecting them to grinding. The following procedure was employed: Into the trachea of freshly removed lungs a small quantity of saline was introduced and washed to and fro by means of a syringe. On withdrawal from the

lungs this fluid appeared milky and contained many epithelial cells. As is indicated in Table I, such fluids showed a relatively high virus-combining titer.

Experiments were carried out to determine what influence variation in the concentrations of virus and lung tissue, with respect to each other, had upon

TABLE I  
*The Capacity of Suspensions of Various Normal Tissues to Combine with PVM*

Saline suspension of normal tissue 10 per cent	Agglutination vs. mouse RBC in presence of 3 units of free PVM							PVM-combin- ing titer of sus- pension
	Dilution of tissue suspension							
	4*	8	16	32	64	128	256	
Mouse lung.....	0	0	0	0	0	2	3	64*
“ bronchial washings.....	0	0	0	2	3	3	3	16
“ kidney.....	3	3	3	3	3	3	3	0
“ brain.....	3	3	3	3	3	3	3	0
Hamster lung.....	0	0	0	0	0	2	3	64
“ kidney.....	3	3	3	3	3	3	3	0
“ brain.....	3	3	3	3	3	3	3	0
Cotton rat lung.....	0	0	3					8
“ “ kidney.....	3	3	3					0
“ “ brain.....	3	3	3					0
“ “ liver.....	3	3	3					0
“ “ spleen.....	3	3	3					0
“ “ muscle.....	3	3	3					0
Rabbit lung.....	0	2	3					4
Guinea pig lung.....	0	2	3					4
Human lung.....	0	2	3					4
Chick embryo lung.....	3	3	3					0
Whole chick embryo.....	3	3	3					0
Whole duck embryo.....	3	3	3					0

\* Reciprocal of dilution or titer.

the binding of virus by the lung tissue component. The results are presented graphically in Fig. 1. It was found that, as the concentration of lung tissue was increased, the quantity of virus bound also increased and it appeared that the two variables were directly related, one to the other, according to multiple proportions.

*Virus-Combining Capacity of Infected Tissues.*—Curnen, Pickels, and Horsfall (5) showed that it is possible by means of a special technique to obtain free infectious PVM from infected mouse lungs; if such lungs are ground, however,

no free virus can be demonstrated directly. It appears evident, therefore, that suspensions of infected mouse lungs, like those prepared from normal animals, are capable of combining with the virus. It was of interest to determine whether suspensions of infected lungs, in which a certain proportion of the virus-combining component was already combined with PVM, would show a virus-combining titer significantly lower than that of suspensions of normal lungs. With experimental conditions identical with those described above it was found

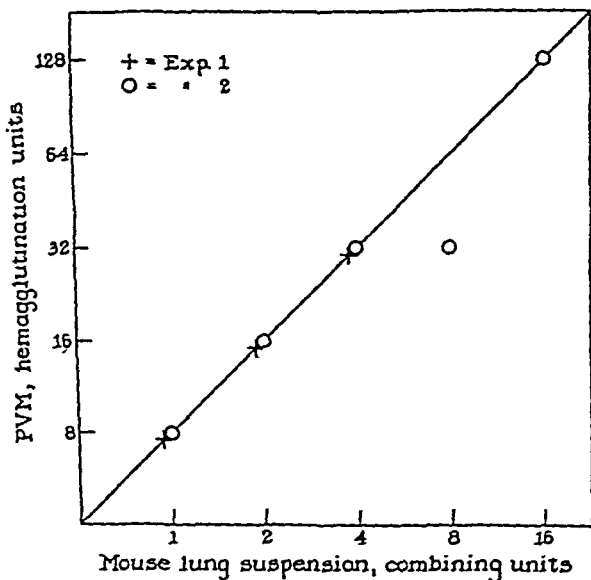


FIG. 1. Relationship between quantity of combining component and quantity of virus bound. One combining unit = the amount of mouse lung suspension which will bind 8 hemagglutination units of PVM.

that the combining capacities of normal and infected lungs were not demonstrably different. This was true even if the infected lungs were removed at times following inoculation when the virus titer was maximal. It was found that suspensions of infected lungs were capable of combining with approximately 10 times more virus than was present in them. It appears, therefore, that only about 10 per cent of the virus-combining component present in mouse lungs is combined with PVM in suspensions of infected lungs.

These results made it even more difficult to understand how free (*e.g.*, uncombined) virus could be present in intact lungs which contain such a relatively large excess of the tissue component capable of combining with PVM. That free virus actually can be obtained from slices of perfused infected lungs by the centrifugation technique described previously (5) was readily confirmed. To explain these results it seemed necessary to assume that the free virus and tissue

component are separated spatially from one another and have in the intact lung no opportunity for contact. If this were true, it appeared probable that free virus would be present in the bronchi. To test this possibility lungs were removed from infected mice on the 6th day after inoculation when the virus titer was maximal. One-half cc. of saline was introduced into the bronchial tree through the trachea, promptly removed, and tested for the presence of virus. It was found that virus was present in the recovered fluid and was in the free state. Such fluid may show a hemagglutination titer of 1,000 to 2,000 which is higher than the titer usually obtained with 10 per cent suspensions of ground lungs following appropriate heating. It has not been possible to devise any procedure which would yield evidence as to the state of PVM in cells infected with the virus.

*Insusceptibility of Avian Embryos to Infection.*—The results of previous experiments (4, 6) suggested that the chick embryo is not susceptible to infection with PVM. It was of importance to investigate this further because, if infection could be established in chick embryos which possess no virus-combining component, it seemed probable that a rich source of free PVM would become available. Since duck embryos also do not possess virus-combining component, they were studied in a similar manner.

Combined virus as well as free infectious virus in suspensions which possessed hemagglutination titers of 1:256 to 1:512 was inoculated in chick embryos. In some experiments the virus was injected into the allantoic fluid of embryos ranging in age from 9 to 13 days. The quantity injected was varied from 0.1 to 1.0 cc. The virus content of the allantoic fluid of inoculated embryos was measured every 2nd day for periods as long as 8 days after inoculation; both hemagglutination tests and infectivity tests in mice were employed. In other experiments the virus was injected into the amniotic fluid of 9 to 11 day embryos in amounts of 0.1 cc. and the virus content of the amniotic fluid as well as of the embryo lungs was measured every 2nd day for 8 days. In still other experiments 0.5 cc. amounts of virus were injected into the yolk sac of 6 to 7 day embryos and the virus content of the yolk sac, whole embryo, embryo lung, and amniotic fluid was measured at intervals up to 8 days after inoculation. For inoculation of duck embryos free infectious virus, which had a hemagglutination titer of 1:256, was used. Embryos, 11 to 13 days of age, were given either 0.5 cc. by the allantoic route or 0.1 cc. by the amniotic route. Allantoic fluid and amniotic fluid as well as lung tissue from duck embryos were tested for the presence of virus by the hemagglutination technique at intervals for 8 days after inoculation.

The results of the inoculation of chick and duck embryos with PVM were unequivocal; in no instance was any evidence of infection obtained. It appears, therefore, that neither free virus nor combined virus is capable of inducing infection in the chick or duck embryo, irrespective of the quantity of virus inoculated or the route by which it is injected.

*Centrifugation of Virus-Combining Component.*—Centrifugation experiments were carried out to determine whether it was possible to separate, in high gravitational fields, the virus-combining component from lung tissue particles. It

was found that, following centrifugation of lung suspensions at 8,000 R.P.M. for 10 minutes, a large proportion of the binding component was sedimented together with cell fragments. By spinning at 15,000 R.P.M. for 30 minutes all or almost all of the binding component was sedimented. Curnen and Horsfall (4) showed that, after hemolysis of mouse red blood cells, all of the combining material remained with the stromata and could be sedimented by high speed centrifugation. These findings were readily confirmed. It appears, therefore, that the tissue component responsible for combination with PVM either consists of particles of appreciable size or is closely associated with small cell fragments from which it is not separated by centrifugation. It has not been possible to obtain any evidence indicating that the tissue component is actually soluble in either water or saline solution.

TABLE II  
*The Effect of Enzymes on the PVM-Combining Component of Mouse Lungs*

Saline suspension of tissue 10 per cent	Treated for 3 hrs. at 37°C. with	Agglutination vs. mouse RBC in presence of 8 units of free PVM							
		Dilution of mouse lung suspension							
		4*	8	16	32	64	128	256	512
Mouse lung	Saline	0	0	0	0	0	2	3	3
" "	Ribonuclease	0	0	0	0	0	2	3	3
" "	Desoxyribonuclease	0	0	0	0	0	3	3	3
" "	Trypsin	3	3	3	3	3	3	3	3

\* Reciprocal of dilution.

*Enzymatic Destruction of Virus-Combining Component.*—To obtain further information regarding the nature of the binding substance present in mammalian lungs, the effect of various enzymes on the component was tested. Mouse lung suspensions were mixed with various enzymes at a concentration of 0.1 mg. per cc. and the mixtures were held at 37°C. for 3 hours. The virus-combining capacities of the treated suspensions were tested according to the technique described above. The results are shown in Table II. It was found that neither crystalline ribonuclease<sup>1</sup> nor desoxyribonuclease<sup>1</sup> had any effect upon the component. Because, as was previously pointed out (4), trypsin<sup>1</sup> is itself capable of causing agglutination of RBC obtained from certain mice, it was necessary to discover some means of preventing this action if satisfactory tests were to be carried out. Fortunately, it was found that trypsin inhibitor<sup>1</sup> (9) prevents agglutination of mouse RBC by trypsin, and, therefore, inhibitor was added to lung suspensions following treatment with trypsin. Under these

<sup>1</sup> These materials were kindly provided by Dr. Maclyn McCarty of The Rockefeller Institute for Medical Research.

conditions it was possible to show that treatment with crystalline trypsin caused lung suspensions to lose all capacity to combine with PVM. The results of these experiments indicate that the activity of the combining component is destroyed by trypsin, and suggest that protein may be an important constituent of the component.

*Dissociation of Virus-Tissue Component Complex by Antibody.*—Results obtained previously by Curnen and Horsfall (4) indicate that spontaneous dissociation of the combination between PVM and the lung tissue component does not occur. In their experiments suspensions of infected lungs which contained a large excess of combining component were used. It appeared possible that dissociation of the complex might actually occur but be masked because the virus could be bound again by lung tissue particles other than those with which it had combined originally. Further experiments were carried out to test this possibility.

To suspensions of infected mouse lungs which contained combined virus, free virus was added in a quantity just sufficient to unite with all the excess combining component. In addition, suspensions of mouse RBC were mixed with amounts of free virus sufficient to saturate their combining capacity. These mixtures were held either at 37 or 42°C. for periods as long as 4 days. At intervals aliquots were removed, centrifuged, and tested for the presence of free virus by the hemagglutinating technique.

It was found that in no instance was demonstrable free virus released from suspensions of mouse lungs or RBC, the binding capacity of which had been saturated with free virus. It appears, therefore, that spontaneous dissociation of the complex formed by combination of PVM and the combining component does not occur.

Experiments were carried out to determine the relative affinities for the virus of the combining component and specific antibody against PVM. It will be recalled that the amount of virus which is combined with the lung tissue component can be determined by heating a suspension appropriately and measuring the hemagglutination titer of the released virus. It was found also that the amount of virus which is combined with antibody, when the virus is mixed with immune hamster serum, can be estimated in a similar manner after heating of the mixture at 70°C. for 1 hour, provided that the antibody is not in too great excess.

Two series of experiments were performed: In the first series a neutral mixture of free virus and hamster immune serum was added to a suspension of normal mouse lung tissue particles which had been washed in the usual manner. After incubation at 37°C. for 1 hour, the lung tissue particles were separated from the mixture by centrifugation, washed with saline 3 times, resuspended in saline, and heated at 70°C. for 30 minutes. The suspension was then tested for the presence of virus by the hemagglutination technique. In no instance was virus demonstrable. In the second series of experiments a suspension of similar lung tissue particles which had been saturated with free virus was mixed with hamster immune serum and incubated



at 37°C. for 1 hour. The lung tissue particles were then centrifuged out. The supernate was heated at 70°C. for 1 hour and the presence of virus was determined by the hemagglutination technique. In every instance virus was demonstrated.

The results of typical experiments, shown in Table III, indicate that when the virus is combined with antibody it is not capable of combining with lung tissue particles but, if the virus is combined with lung tissue particles, it can be caused to dissociate from them by specific antibody with which it then combines. It appears, therefore, that the attractive forces between the virus

TABLE III  
*The Dissociation of Combined PVM by Specific Antibody against the Virus*

Mixture		Centrifuged at 12,000 R. P. M.	Heated at 70°C. for	Agglutination vs. mouse RBC								Titer of free PVM
				Dilution of mixture								
				2*	4	8	16	32	64	128	256	
Combined PVM, (lung tissue particles saturated with virus)	Saline	Supernate	60 min.	0	0	0	0	0	0	0	0	0
	" "	Sediment, washed and resuspended	30	3	3	3	2	2	±	0	0	32*
" "	Anti PVM serum (hamster)	Supernate	60	3	3	2	2	0	0	0	0	16
" "	" "	Sediment, washed and resuspended	30	0	0	0	0	0	0	0	0	0

\* Reciprocal of dilution or titer.

and specific antibody are stronger than those between the virus and the combining component.

*Virus-Combining Capacity of the Intact Lung.*—The experiments described above were all carried out *in vitro* but the chief objective of this study was to learn more of what occurs in the lung of a living animal after the intranasal inoculation of PVM. It seemed of importance to determine whether, following inoculation, virus was bound by the intact lung, and whether this occurred only if free virus was given or would occur even when combined virus was inoculated. It was also of interest to learn the rate of the interaction between virus and intact lung. Hirst's (10) studies with influenza virus in ferret lungs had shown that important information could be obtained by

techniques employing intact surviving lungs. Similar studies were undertaken with PVM in mouse or hamster lungs. The very small size of the lungs of these animals necessitated some modifications in procedure.

Because the mouse lung is so small, most of the experiments were carried out with surviving hamster lungs. To prevent confusing effects which might be caused by the presence of either specific antibody or red blood cells, each lung was carefully perfused with saline *in situ* before removal. One cc. or less of virus suspension was injected into the lung through the trachea; the trachea was then ligated and the distended lung held for various periods at 37°C. Fluid was then recovered from the lung and its virus content measured by the hemagglutination technique. It was found that the largest amount of fluid was recovered when the trachea

TABLE IV  
*The Capacity of Intact Hamster Lungs to Bind Free and Combined PVM*

Virus introduced via trachea in hamster lungs	Time virus was held in lungs at 37°C.	Agglutination vs. mouse RBC										Hemagglutination titer
		Dilution of fluid recovered from lung										
		2*	4	8	16	32	64	128	256	512	1024	
	hrs.											
Free PVM	0	4	4	4	3	3	3	2	2	2	0	512*
“ “	0.5	4	4	3	3	3	3	2	0	0	0	128
“ “	1.0	2	2	±	0	0	0	0	0	0	0	4
“ “	1.5	0	0	0	0	0	0	0	0	0	0	0
Combined PVM	0	4	4	3	3	3	3	3	3	2	0	512
“ “	1	4	4	3	3	3	3	3	3	2	0	512
“ “	2	4	4	3	2	2	2	2	0	0	0	128
“ “	4	3	3	2	±	0	0	0	0	0	0	8
“ “	6	0	0	0	0	0	0	0	0	0	0	0

\* Reciprocal of dilution or titer.

was cut off, and the lung placed in a lusteroid tube and centrifuged at high speed. With this procedure about 75 per cent of the fluid which had been injected could be recovered and results obtained with individual lungs compared well, one with another.

The results when free virus was introduced into the hamster lung are shown in Table IV. It was found that free virus was removed from the fluid and bound promptly by the lung. In 1 to 1.5 hours all of the virus which had been injected was bound and none could be demonstrated in the fluid recovered from the lung. Experiments carried out with either heat-released virus or with free infectious virus gave identical results. In Table IV also are shown the results which were obtained when combined virus was employed. It was found that, given sufficient time, combined virus also could be bound by the lung but that to obtain comparable removal of such virus from the fluid introduced a time interval at least four or five times longer was required with com-

bined virus than with free virus. Closely similar results were obtained in experiments with intact mouse lungs.

In order to exclude the possibility that the hemagglutinating capacity of PVM was destroyed in the intact lung, it was necessary to show that the virus could be released from the lung tissue following its introduction *via* the trachea. In an accompanying paper (11) it is shown that concentrated lung suspensions possess the capacity to cause marked reduction in the hemagglutination titer of PVM and that this adverse effect can be completely prevented by the addition of iodoacetamide to such suspensions. When intact lungs which apparently had bound virus were ground and heated appropriately, irregular results were obtained in hemagglutination tests and in no instance were the titers of the heat-released virus as high as was to have been expected. However, when iodoacetamide (0.01 M) was added to suspensions of such lungs, consistent results were obtained following heating and the observed hemagglutination titers corresponded reasonably well with those to be expected from theoretical considerations. Thus, it appears that the intact hamster or mouse lung is capable of binding PVM after introduction of the virus through the trachea, and that virus so bound can be released from the lung tissue under appropriate experimental conditions.

*Dissociation of Combined Virus in the Lung.*—The results of experiments on the binding of PVM in surviving lungs showed that a much longer time was required for binding when combined virus was injected than was necessary when free virus was employed. That so much time was needed before combined virus was bound suggested that a step-wise mechanism might be operative and it seemed logical to think that the first step might be dissociation of the virus-tissue component complex. As shown above, the results of numerous experiments have indicated that spontaneous dissociation of the complex does not occur. To determine whether combined virus did undergo dissociation following its introduction into the lung, various experimental procedures were employed.

The following procedure yielded the most decisive results: To a suspension of mouse lungs infected with PVM was added sufficient free virus to completely saturate the combining capacity of the tissue. One cc. of the mixture was then introduced into a surviving hamster lung *via* the trachea. After incubation at 37°C. for 1 hour, the fluid was recovered from the lung and placed in a test tube which was held at 37°C. in a water bath. At intervals aliquots were removed, centrifuged, and tested for the presence of free PVM by the hemagglutination technique.

The results of such an experiment are shown in Table V. It was found that virus was slowly released from combination during incubation of the fluid recovered from the lungs. The time required for maximal release of free virus varied markedly from one experiment to another and ranged from 2 to 20 hours. These results indicated that fluid obtained from the intact lung could

cause dissociation of the virus-tissue component complex and suggested that one or more enzymes present in the lung might be responsible for the dissociation.

To obtain information concerning the possibility that an enzyme might release virus from combination with the lung tissue component, attempts were made to obtain such release by means of highly purified enzymes. As above, crystalline trypsin is capable of destroying the virus-combining capacity of

TABLE V

*The Release of Free PVM from Combined Virus in the Intact Hamster Lung and by the Action of Trypsin*

Virus	Treatment	Time recovered fluid was held at 37°C.	Agglutination vs. mouse RBC						Titer of free PVM
			Dilution of fluid recovered from lung						
			2*	4	8	16	32	64	
Combined PVM, (lung suspension saturated with virus)	Held 1 hr. in hamster lung	hrs.							
		0	0	0	0	0	0	0	
		2	2	0	0	0	0	2*	
		4	2	2	2	0	0	8	
		20	3	2	2	2	0	0	16
Mixture		Time mixture was held at 37°C.	Dilution of virus-enzyme mixture						
2*	4		8	16	32	64			
Combined PVM, (lung suspension saturated with virus)	Trypsin, 0.01 mg./cc.	hrs.							
		0	0	0	0	0	0	0	
		1	3	3	3	2	±	0	16
		1.5	3	3	3	2	2	±	32
		2	2	2	2	±	0	0	8
4	0	0	0	0	0	0	0		

\* Reciprocal of dilution or titer.

lung tissue suspensions, and consequently trypsin was used in the present experiments. Unfortunately, trypsin possesses properties which required careful control; not only may it cause agglutination of mouse red blood cells but also, as shown previously (4), it can cause destruction of the virus itself. The tendency to cause agglutination of mouse RBC was inhibited, as described above, by the addition of trypsin inhibitor immediately before hemagglutination tests were carried out. When relatively small amounts of the enzyme were added to lung tissue suspensions which had been saturated with virus, and aliquots were tested at frequent intervals for the presence of free virus by means of the hemagglutination technique, reproducible results were obtained. As is shown by the results also presented in Table V, crystalline trypsin appears to be capable of releasing PVM from combination with the lung tissue com-

ponent. Maximal hemagglutination titers were obtained after incubation of the mixtures for 1 to 1.5 hours at 37°C. Further incubation resulted in a progressive decrease in hemagglutination titer and after 4 hours' incubation free virus was no longer demonstrable. The decline in titer with time is due, in all probability, to enzymatic destruction of the virus. It appears that the results of the action of trypsin, with respect to the release of PVM from combination, resemble those obtained with fluid recovered from intact lungs and thereby lend support to the hypothesis that the latter results may be attributable to the action of enzymes present in the lung.

#### DISCUSSION

The results of the experiments carried out in this study indicate that the lungs of each of the mammalian species examined, but not avian embryos as such, contain a component which combines firmly with PVM. The capacity of mammalian lung to combine with PVM appears to be dependent upon the presence of a specific tissue substance which possesses an affinity for the virus sufficiently striking as to be exceeded only by that of specific antibody against PVM. That combination between the virus and the lung tissue component is not merely the result of non-specific adsorption of the virus to tissue particles is demonstrated clearly by the fact that suspensions of mammalian tissues other than the lung are incapable of binding PVM. With the exception only of the erythrocytes of mice and hamsters the combining component appears to be present solely in mammalian lungs and can be demonstrated as readily in the intact lung as in lung tissue suspensions.

The complex formed by combination between the lung tissue component and PVM is sufficiently firm and stable as not to dissociate except in the presence of specific antibody against the virus or following treatment by procedures which destroy the combining capacity of the tissue component; *i.e.*, heat (4), alkali (6), or digestion with trypsin. The finding that crystalline trypsin, but not other enzymes, is capable of causing dissociation of the complex with release of free virus suggests that protein may be an essential constituent of the combining component.

Evidence has been obtained which strongly suggests that the virus-binding component present in mammalian lungs may play a decisive rôle in the initiation of infection with PVM. Striking correlations, both qualitative and quantitative in kind, have been found between the distribution of the combining component among various animal species as well as among their several organs and susceptibility to infection with the virus. It will be recalled that PVM is, according to present evidence, strictly pneumotropic; in no instance has it been possible to establish infection in an animal by any route other than the intranasal and in no instance has it been possible to recover the virus from any tissue other than the lung. It appears, therefore, that the only mammalian

organ which is capable of becoming infected with PVM is that organ which, as distinct from others, contains a tissue component capable of combining with the virus. Moreover, different animal species are susceptible to infection with PVM in different degree. Mice and hamsters appear to be most susceptible and both chick and duck embryos appear to be completely insusceptible to infection with the virus. These species differences in susceptibility are directly correlated with the quantity of combining component present in the lungs of the several species as is evident from the finding that the lungs of mice and hamsters contain much of the component, those of other mammalian species considerably less, and those of avian embryos none.

If, as appears probable, the first step in the initiation of an infection with PVM is combination between the virus and the lung tissue component present at the periphery of susceptible cells, it may seem paradoxical that combined virus should be equally as infectious as free virus. Evidence obtained in this study seems adequate to overcome this objection to the hypothesis. It appears that combined virus can be dissociated by a substance present in the intact lung with the result that free virus is released. Inasmuch as a similar dissociation can be affected by trypsin, it seems probable that dissociation occurring in the lung may be the result of enzymatic action and that such dissociation also occurs in the lung of the living animal. Under these circumstances a preliminary step in the establishment of infection with combined virus would be splitting of the virus-tissue component complex in the inoculum. The free virus, released in this manner, could then recombine with the lung tissue component at the periphery of the susceptible cells in the lung of the living animal. The infectiousness of combined virus, therefore, appears not to be an objection to the present concept regarding the significance of the virus-combining component with respect to susceptibility to and the initiation of infection with PVM.

Inasmuch as mouse lungs infected with PVM contain such a large excess, of the tissue component which combines with PVM,—at least 10 times more than is required to bind all of the virus,—it may appear strange that it is possible to obtain free virus from such lungs by any procedure. It should be emphasized that free infectious virus has been obtained solely from intact lungs or from lung slices, and not from ground lung suspensions. Moreover, although bronchial washings from normal mouse lungs contain the combining component, the concentration is not high and the quantity present is not sufficient to combine with more than about 10 per cent of the free virus present in similar washings from infected mouse lungs. It appears evident that much free virus is present in intact infected lungs and that an appreciable amount of virus in the free state finds its way into the bronchial lumina. It seems probable that much the greater part of the combining component is inaccessible to the virus in the intact infected lung, especially to those virus particles which during the

course of infection are released into the bronchi, and that, because of this, free virus can be obtained from such lungs provided the tissue is not ground.

#### SUMMARY

Evidence has been obtained which indicates that the lung tissues of mammalian species susceptible to infection with PVM contain a specific component which combines with the virus. The concentration of this tissue component appears to be directly proportional to the susceptibility of the species; in its absence infection with PVM cannot be established. The available evidence suggests that the presence of the virus-combining component in lung tissue may play a decisive rôle in the initiation of infection with this pneumotropic virus.

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# TRANSMISSION OF EPIDEMIC GASTROENTERITIS TO HUMAN VOLUNTEERS BY ORAL ADMINISTRATION OF FECAL FILTRATES

BY IRVING GORDON, M.D., HOLLIS S. INGRAHAM, M.D.,  
AND ROBERT F. KORNS, M.D.

*(From the Division of Laboratories and Research and the Division of Communicable Diseases, New York State Department of Health, Albany)*

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Numerous outbreaks of gastroenteritis occurred in New York State institutions during the fall and winter of 1946-47. The disease was characterized by sudden onset, with profuse diarrhea, usually accompanied by vomiting, and by a lack of fever. Symptoms ordinarily persisted for about 3 days. Bacteriologic examinations failed to reveal agents known to cause enteric disease and postmortem investigations also failed to indicate the nature of the etiologic agent. A search was therefore undertaken for a non-bacterial pathogen. Since preliminary animal experiments were unsuccessful, human volunteers were utilized. The illness was reproduced and transmitted in series in volunteers and the responsible agent was shown to be filtrable. Oral administration of stool suspensions or throat washings induced the disease, but inhalation of nebulized throat washings did not. Volunteers who were fed material from the third embryonated egg passage likewise remained asymptomatic. The present report describes these experiments.

## *Epidemiologic and Clinical Features of the Disease*

The first of the series of outbreaks with which this report deals was noted in the summer and fall of 1946 in New York City hospital populations. Later the disease occurred in several large mental hospitals near the metropolitan area, and during the course of the winter, there were sizable outbreaks in at least thirteen of the twenty-four New York State mental institutions and in two prisons. Similar episodes are known to have affected a number of mental hospitals in Massachusetts (1).

The specific epidemic from which material was obtained for transmission experiments occurred at the Marcy State Hospital, near Utica, New York. The epidemic was typical, and thus can serve to describe the outbreaks in all the institutions. The disease appeared on December 16, 1946, and was at first largely confined to one building, where in a 2 week period two hundred sixty-one of the five hundred eighty-nine patients suffered from the clinical disease. It gradually spread to each of the other buildings and by January 31, 1947, when the last case was noted, five hundred eighty-nine cases with seven deaths had

occurred among the two thousand, six hundred twenty-three inmates and one hundred fifteen cases among the three hundred fifty-four employees. The deaths occurred in aged or deteriorated mental patients. Within the limitation of the institution population there seemed to be no age or sex selection in the attack rate. The pattern of spread bore no evident relationship to the distribution of milk, food, or water, and sanitary inspection failed to reveal faults that could have accounted for the outbreak.

Thus, epidemiologic investigation indicated that these outbreaks were not from a common source but spread through direct person-to-person contact. Outbreaks occurred during the period from August, 1946, through June, 1947, with some tendency for grouping during December and January. The concentration of the disease in institution populations may be more apparent than real. There is less precise evidence that the general population of upstate New York was also affected. Field studies did not indicate whether the spread occurred through the gastrointestinal or the respiratory routes.

Clinically the patients had a rapid, sometimes dramatic onset of nausea, vomiting, or diarrhea. These happenings, almost always accompanied by anorexia, occurred singly or in combination. Some patients never developed diarrhea. The vomiting was frequently unexpected, sudden, and violent. Abdominal cramps, borborygmi, headache, dizziness, and malaise were common symptoms. Fever was usually absent, or, if present, slight. Recovery ordinarily ensued in 48 to 72 hours, but at times required a week. Respiratory symptoms were not evident. Leucocyte counts were within normal limits except in dehydrated patients. The stools were copious and watery, often justifying description as "pea soup." Blood was rarely seen. Stool specimens from a total of thirty-four patients were examined for bacterial pathogens by the methods given below; none were found.<sup>1</sup>

### *Methods of Study*

*Subjects.*—Twenty-eight men 21 to 25 years of age and six between 16 and 21 years of age volunteered to participate in the study. Only the adult volunteers were inoculated with presumably infective material; the minors received autoclaved inoculum. Thorough studies were carried out on each adult subject before he was accepted. These included physical examination, chest roentgenogram, blood count, urine examination, sedimentation rate, pharyngeal culture, and multiple stool cultures. Each volunteer was free of recognized infectious or organic disease. None had had diarrhea, nausea, or vomiting during the previous year. It was impossible to restrict selection to individuals who had not been exposed to epidemic diarrhea, since a sharp outbreak of the disease had occurred 2 months previously in an institution

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<sup>1</sup> Identical results were obtained by Dr. George C. Bower, who examined by rectal swab on *Shigella-Salmonella*-agar plates stools from five hundred eighty nine patients at Marcy State Hospital, and by Dr. W. R. Strutton who examined stools from over six hundred patients at Rockland State Hospital. Both of these hospitals had recently done rectal swab surveys designed to discover all carriers of *Shigella* organisms.

from which many of the volunteers were drawn. Men who had suffered even trivial symptoms, however, were not accepted.

*Precautions for Isolation.*—The isolation quarters consisted of two rows of eleven cubicles separated by a central corridor. The corridor was isolated from the rest of the institution by a draft-proof door. Each cubicle had an outside window and a draft-proof door to the corridor; there were no other openings. The volunteers, quartered one to a cubicle, remained in them for the duration of each experiment.

Each cubicle had sink and toilet facilities and each volunteer had his own set of dishes, which he washed himself. Food was dispensed from a cart without touching the dishes. Only one door was opened at a time. The precautions of a contagious pavilion were observed. Only the personnel conducting the experiment entered the quarters.

*Collection and Preparation of Inocula.*—Three types of inoculum were used: fecal suspensions, throat washings, and chick embryo tissues and fluids. All were frozen within 5 to 15 minutes after collection and stored in a dry-ice chest at approximately  $-70^{\circ}\text{C}$ . Specimens were thawed at  $37^{\circ}\text{C}$ . when required for inoculation.

All stool specimens used as inoculum were watery and further dilution was unnecessary. They were centrifuged for a total of 75 minutes at 3000 R.P.M. in a refrigerated horizontal centrifuge (radius 15.5 cm.) which was halted once to remove the supernatant fraction. The final supernatant, which constituted the inoculum, was clear.

Throat washings were collected by having the donors gargle beef-infusion broth containing 10 per cent horse serum. Subsequent clarification by centrifugation was identical with that of stool specimens.

The tissues and fluids harvested from embryonated eggs were chorioallantoic membrane, amniotic membrane, yolk sac, amniotic fluid, and allantoic fluid. Not all of these materials were harvested from each egg. Tissues were ground in a mortar with alundum and made into a 10 per cent suspension (by weight) with allantoic fluid or a mixture of amniotic and allantoic fluids. After light horizontal centrifugation (500–1000 R.P.M.) for 10 minutes, the supernatant was employed as inoculum.

Inocula were kept on cracked ice if they were to be used within several hours after preparation; for longer periods they were refrozen, stored in the dry-ice chest, and thawed immediately before use.

*Filtration.*—Material to be filtered was drawn by light suction through Corning sintered glass "UF" filters known to hold back bacteria (streptococci, staphylococci, *Bacterium coli*, *Bacterium enterocoliticum*). The filter surfaces were prepared with broth before filtration. Duplicate thioglycollate, aerobic and anaerobic sterility broth, and blood-agar cultures of the filtrates were incubated at  $37^{\circ}\text{C}$ . for 1 month. All were sterile.

*Methods of Inoculation.*—Two routes of inoculation, oral and respiratory, were used. Inoculum administered by the oral route was fed in double gelatin capsules or drunk from a paper cup. A penicillin nebulizer<sup>2</sup> was employed to inoculate by the respiratory route. The system was a closed one; the volunteers' lips were shut around the opening of the nebulizer and they exhaled into a rebreathing bag. Compressed air or compressed nitrogen was used as a source of pressure to nebulize the inoculum. Inoculations by nebulizer were conducted in an area distant from the isolation quarters, either in the open air or in an entry-way for shelter against the weather.

*Observations on Experimental Subjects.*—The volunteers were seen at least once each day and symptoms and signs of illness were recorded in a uniform manner. White blood counts, differential counts, specific gravity determinations of the blood and plasma (2), and sedimentation rates (3) were done, and chest roentgenograms were prepared if the subject had been

<sup>2</sup> Distributed by the Oxygen Equipment Company, New York City.

inoculated by inhalation. Medication was limited to the use of barbiturates and codeine. Specimens of serum were collected before inoculation and at intervals thereafter. They were examined for antibodies to influenza viruses A and B (4) and for cold agglutinins (5) since an epidemic of influenza and other respiratory disease occurred in New York State during the course of the experiments. No rises in antibody levels were detected.

*Stool Cultures.*<sup>1</sup>—Stool cultures from patients with diarrhea were made with fresh or glycerolated material. Endo, cosin-methylene blue, bismuth sulfite, and *Shigella-Salmonella* (bile salt) agar plates were streaked with each specimen, whether from a case of natural or experimental infection, and in some instances tetrathionate broth was also inoculated.

*Other Technical Procedures.*—Technical procedures not specifically mentioned were those of the Division of Laboratories and Research (6).

### *Experiments on Transmission*

Two experiments were done. The first, a preliminary study, was intended to determine the relative activity of stools and throat washings. The disease was reproduced in three volunteers who swallowed unfiltered stool suspension, but not in three who inhaled throat washings. The second experiment was designed to demonstrate whether the agent was filtrable; to extend the investigations on the infectivity of throat washings; and to test material from embryonated eggs previously inoculated with infective stool. Twenty-two volunteers were employed in these studies, in the course of which the opportunity was presented of reinoculating recovered subjects, thus obtaining limited data on active immunity. Finally, a group of six boys were fed autoclaved stool as a control observation.

*Donors.*—Two patients at Marcy State Hospital who had characteristic disease were selected as donors. Case reports follow.

*J. H.*, a 35 year old female, had been in good physical health until early in the afternoon of January 7, 1947, when she experienced nausea and abdominal discomfort, quickly followed by vomiting and diarrhea. During the night she vomited six times and had ten watery stools. She complained of anorexia, dizziness, headache, weakness, and feverishness, was slightly dehydrated, had a temperature of 100.2°F., and a leucocyte count of 13,850. Physical examination was negative. Treatment was symptomatic. On the next day her symptoms abated, her temperature was 99.4, and the leucocyte count 10,000. She was completely recovered by the end of the 3rd day. Slight albuminuria, the only abnormal urinary finding, was noted during the period of dehydration and persisted for a few days after recovery. Stool specimens and throat washings were collected approximately 5 hours after onset.

*C. W.* was a 46 year old male also in good physical health, whose illness began suddenly on January 5, 1947, with diarrhea followed by nausea, vomiting, giddiness, weakness, and abdominal cramps. These symptoms diminished somewhat on the following day but during the 2nd day of illness he vomited once, had five stools, his temperature was 99.6, and the leucocyte count was 7,850. Physical examination showed slight edema of the uvula and hyperemia of the pharynx. Anterior cervical and axillary lymph nodes were palpable but not tender. He was completely recovered on the 3rd day after onset. Stool specimens and throat washings were collected approximately 48 hours after onset.

<sup>1</sup> The stool cultures were examined by Miss Marion B. Coleman.

Neither of these patients had evidence of acute respiratory infection. Repeated throat cultures revealed none of the commonly recognized pathogens. Roentgenograms of the chest were normal. Bacteriologic and parasitologic examination of multiple stool specimens was negative for known pathogens. Blood cultures, taken at the time the stools were collected, were sterile.

### *First Experiment*

Clinical observation of six volunteers was begun 3 days before each of three was fed 3.6 ml. of a pool of equal parts of unfiltered stool suspension from J. H. and C. W. All three developed characteristic gastroenteritis; case 1 had an incubation period of  $1\frac{1}{2}$  days; case 2,  $2\frac{1}{2}$  days; case 3, 5 days. Their illnesses closely resembled those of the donors but symptoms were considerably milder in case 3.

The other three volunteers each inhaled approximately 1 ml. of a nebulized mixture of equal parts of throat washings from J. H. and C. W. 3 days after inoculation of the first group. No illness resulted.

During the experiment one of the attendants had a mild episode of diarrhea, unaccompanied by other symptoms, and lasting for a single day. This occurred when cases 1 and 2 first showed signs of illness. His contact with the volunteers during this episode lasted only for 3 hours, and then was broken, but there was a history of exposure to a family epidemic of gastroenteritis 2 days previously. It was felt that the attendant's illness was coincidental and unrelated to the illnesses of the volunteers. All six subjects had equivalent contact with the attendant, whereas only those inoculated with stool became ill. The findings in the second experiment supported this opinion.

Bacteriologic examination of the diarrheal stools of the three subjects revealed *Bacterium enterocoliticum* in one specimen from case 2. This organism, which has been isolated under circumstances which incriminate it as a pathogen (7, 8), is also known to occur in the absence of disease (8). Its lack of etiologic significance in this study was proved by the results of the later experiments with filtered stool from the same patient.

### *Second Experiment*

Because of the importance of detecting cross-infection, emphasized by the occurrence of diarrhea in the attendant, the inoculation schedule of the second experiment was staggered so that at all times there was a group of well, susceptible men in the isolation quarters. Of the various inocula employed, each stool filtrate induced typical illness and one pool of unfiltered throat washings caused gastroenteritis when given by mouth; the others failed to cause disease. The first three men to be inoculated were fed stool filtrate on the day following the quarantine of sixteen volunteers (Fig. 1). Six days later, after all three had become ill, nine more subjects were inoculated orally; three with the same stool filtrate, the other six with material from previously inoculated embryonated eggs. Nine days after isolation began four men inhaled unfiltered throat washings, and a group of six, who were newly arrived, were put into isolation. On the following day, three of these were fed diluted

stool filtrate and three drank unfiltered throat washings. Reinoculations with the various materials were also done, as described below. The time distribution of the illnesses and their strict relation to specific inocula indicate that cross-infection did not occur (Figs. 1 and 2).

*Experiments with Stool Filtrates.*—Stool suspensions taken in the first 3 days of illness from cases 1 and 2 of the first experiment were filtered and equal

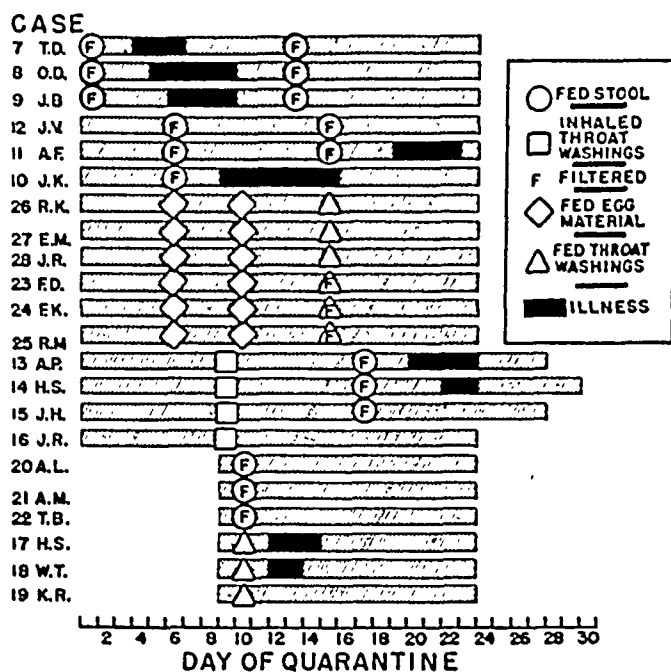


FIG. 1. Duration of quarantine and results of inoculations of each volunteer in the second experiment. Occurrence of gastroenteritis is shown in black.

portions of filtrate pooled. Capsules containing a total of 3.5 to 4.0 ml. of the pool were fed to each of six volunteers; three additional subjects drank 0.05 ml. of the same inoculum diluted in 100 ml. of tap water. The nine men were inoculated on three different days.

Four of the six men who took the larger dose developed typical gastroenteritis, but none of the three receiving 0.05 ml. became ill (Fig. 2). The following report illustrates a moderately severe case:

*Case 10.*—A 24 year old white male was fed eight capsules containing a total of 4.0 ml. of pooled stool filtrate from cases 1 and 2. Approximately 48 hours later he experienced abdominal cramps and borborygmi. Within the next 12 hours the characteristic symptoms of anorexia, nausea, vomiting, and diarrhea developed (Fig. 3). Although symptoms were moderately severe, there was no abdominal tenderness or spasm. The leucocyte count was 7,000. These symptoms continued undiminished for another day during which he became somewhat dehydrated and had a fever (temperature 100.8°F.). Because of the indications in published reports that changes in the central nervous system might be associated with this (9, 10) or

similar (11) disease entities, and that symptoms of vomiting and giddiness might be accounted for on this basis, permission was obtained to perform a spinal tap at this time. The fluid was under normal pressure and there was one mononuclear cell per c. mm., a protein content of 34 mg. per 100 ml., and a normal colloidal gold curve.<sup>4</sup> On the 3rd day of illness the patient was markedly improved, and his symptoms shortly afterward disappeared except for diarrhea; loose stools continued for another 4 days.

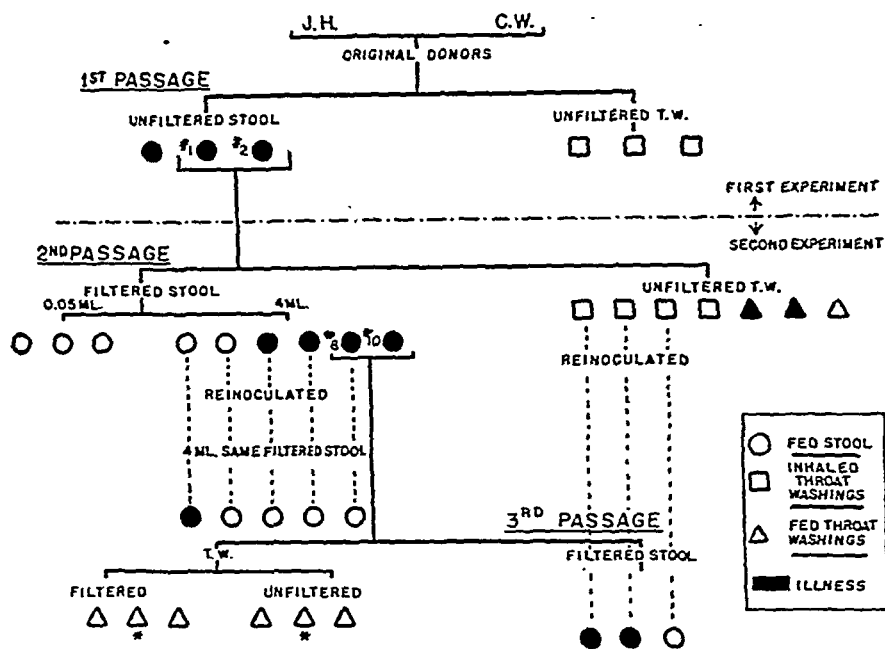


FIG. 2. Passage scheme of the first and second experiments. Occurrence of gastroenteritis is shown in black. Groups of volunteers marked with an asterisk had previously been inoculated with egg material and had remained asymptomatic.

It was previously mentioned that some spontaneous cases of gastroenteritis never exhibited diarrhea. One experimental case was similar.

**Case 7.**—A 21 year old white male, also fed capsules containing 4.0 ml. of pooled stool filtrate from cases 1 and 2, had as his first symptom precipitate vomiting 3 days later followed by moderate nausea and anorexia (Fig. 4). His appetite was never completely lost, however, nor was his nausea continuous. He vomited ten times on the 1st day, six on the 2nd, three on the 3rd, and recovered on the 4th day after onset. During this time he was constipated. Temperature was normal and abdominal examination negative. Reinoculated with a second equal dose of the same inoculum 10 days after onset, he remained well.

To demonstrate whether the gastroenteritis caused by unheated filtrate was due to a heat-resistant component of the stool or perhaps to the ingestion of the

<sup>4</sup>The spinal fluid examination was performed by Dr. Carl Lange.

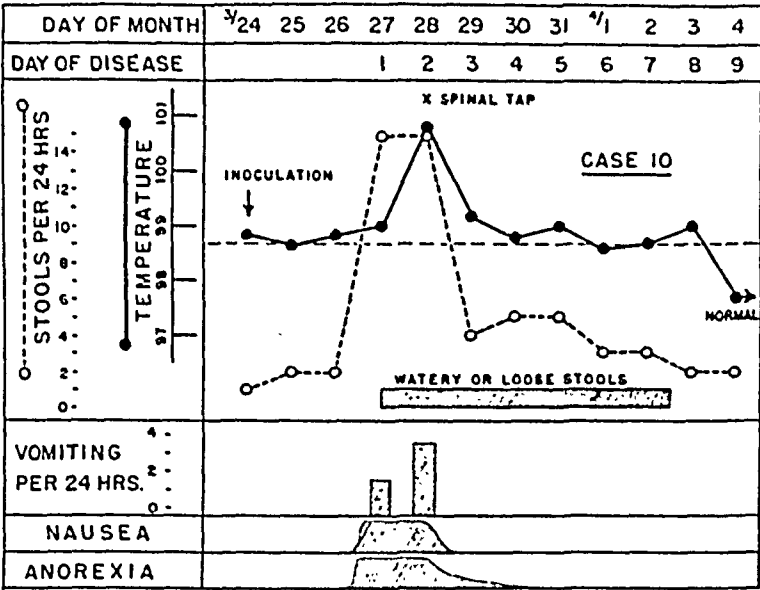


FIG. 3. Case 10. Clinical chart of patient with experimentally induced gastroenteritis.

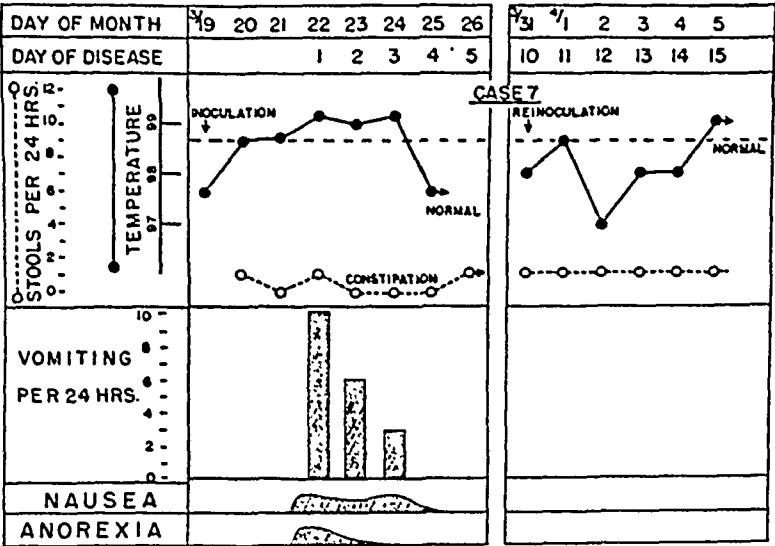


FIG. 4. Case 7. Clinical chart of patient with experimentally induced gastroenteritis; no diarrhea.

capsules as such, a specimen of the pooled stool filtrate from cases 1 and 2 was autoclaved for 20 minutes at 15 pounds' pressure and capsules containing 3.5 ml. of it were fed to each of six boys aged 16 to 21. None subsequently became ill.

The experiments with fecal filtrate showed that the agent was non-bacterial, and that the effective dose probably lay between 0.05 and 4.0 ml. of pooled fil-



trate from cases 1 and 2. There was no certainty that the symptoms were not caused by a filtrable toxin produced by bacteria and a second passage was done to settle this point.

Since dilution of the original 4.0 ml. of inoculum in the voluminous watery stools of the recipients was far greater than the 80-fold difference between the 4.0 ml. effective dose and the 0.05 ml. ineffective dose, a toxin would be diluted beyond the point of causing symptoms. Pooled fecal filtrate from cases 8 and 10, whose illnesses were induced by 4.0 ml. of pooled filtrate from cases 1 and 2, was therefore fed in 6.0 ml. amounts to each of three men\* (Fig. 2). Two (cases 8 and 10) developed typical gastroenteritis, indicating that the agent had multiplied and was not a toxin ingested by the original cases.

TABLE I

*Results of First and Second Inoculations with Pooled Fecal Filtrate from Cases 1 and 2*

Case No.	Results of inoculations		Time	
	First	Second	Between inoculations	Between onset and second inoculation
			days	days
7	Gastroenteritis	No illness	12	10
8	"	" "	12	9
9	"	" "	12	8
11	No illness	Gastroenteritis	9	0
12	" "	No illness	9	0

It was possible to reinoculate five of the six men who were fed 4 ml. of stool filtrate from cases 1 and 2, thus securing limited data concerning the development of active immunity. Since circumstances did not permit delay, the volunteers were reinoculated 9 to 12 days after the first inoculation (Table I). The second dose was 4 to 5 ml. of the same filtrate. Typical gastroenteritis followed in one of the two subjects who had failed to contract the disease the first time. The others remained asymptomatic. A single loose stool was reported by one volunteer but since other symptoms were absent, it was considered to be of little significance. The findings suggest that active immunity may develop within 10 days after onset of the disease (Table I), but results of immunity tests in such a small group with an inoculum of unknown degree of infectivity must be interpreted with great caution.

*Incubation Period and Symptomatology.*—For analysis of the range and average duration of the incubation periods and several of the symptoms, data from the three cases induced by unfiltered stool in the first experiment have been added to those obtained from the seven cases induced by filtered stool in the second experiment. The mean incubation period of the ten

\* These subjects had previously inhaled pooled nebulized throat washings from cases 1 and 2 and had remained well. Further details are given subsequently.

cases was 3 days; it ranged from 1 to 5 days. Vomiting occurred in four of the ten cases, and lasted for no more than 3 days. In contrast, seven of nine cases still had loose stools 4 days after onset, and in one instance this symptom persisted for a week. Borborygmi and abdominal cramps were a constant accompaniment of diarrhea. Leucocyte counts, sedimentation rates, and blood and plasma specific gravities were normal.

*Experiments with Throat Washings.*—The failure of nebulized throat washings to induce gastroenteritis when inhaled by three subjects during the first experiment could have been due either to lack of activity of the inoculum or to non-transmissibility of the disease by the respiratory route. To investigate these points, throat washings were administered orally in the second experiment as well as by inhalation.

Garglings from cases 1 and 2, taken in the first 3 days of illness, *i.e.* during the same period as the stool inocula were obtained, were centrifuged and the pooled supernatant set aside as inoculum. Cultures revealed the following organisms to be present in it: alpha and gamma streptococci, *Staphylococcus aureus*, Gram-negative cocci, and diphtheroids. The latter organisms were not further identified. Four subjects each inhaled a total of 1.5 to 2.0 ml. of nebulized inoculum (Fig. 2). Three others each drank 8 ml. and swallowed capsules containing 2 ml., a total dose of 10 ml.

The four volunteers who inhaled the inoculum remained well and had normal roentgenograms of the chest; and as mentioned previously, when three of them were subsequently fed fecal filtrate, two proved to be susceptible. Of the three men who swallowed 10 ml. of washings, two developed gastroenteritis, the incubation period being 2 days in each instance. These patients had very brief and mild illnesses. One had only diarrhea, the other had anorexia, nausea, and vomiting as well. Both reported "blood" in their stools, but as the reliability of their observations was questionable and as no blood was seen by the personnel conducting the experiments, this point remains in doubt.

Six other volunteers were fed throat washings. The men had been previously inoculated with embryonated egg material, as reported below, and had remained well. Since the pools of washings from cases 1 and 2 were exhausted, a pool from cases 7 and 10 was utilized. Three received 2.0 ml. of unfiltered washings in capsules, the other three were similarly given 3.5 to 4.0 ml. of the corresponding filtrate. None became ill.

*Experiments with Inocula from Embryonated Hens' Eggs*<sup>6</sup>.—A series of blind passages in embryonated hens' eggs (White Leghorn) originally inoculated with stool suspensions by various methods were not productive of unequivocal signs of infection. Irregular deaths of embryos from the 1st to the 9th day after inoculation, a few hemorrhages into various tissues, and edema and ulceration of some chorioallantoic membranes were noted. These phenomena were not reproducible in series and are known to occur in uninfected eggs (12). A virus (*e.g.*, mumps (13)) may grow in eggs in the absence of lesions, however, and since immunologic methods of testing were unavailable, a second series of passages were carried out for the specific purpose of inoculating volunteers with pooled tissue extracts and extraembryonic fluids from selected eggs.

<sup>6</sup> Dr. Lisbeth M. Kraft aided in this phase of the work.

All eggs were incubated at 35-37°C. both before and after inoculation. The inoculum was unfiltered stool from donor J. H., which had been fed to volunteers in the first experiment. That each of these three developed the disease suggested that the inoculum might contain enough of the etiologic agent to inhibit growth through an autointerference effect (14). Therefore, duplicate passage series by each route of inoculation were initiated with both undiluted and  $10^{-1}$  broth dilution of the unfiltered stool suspension. Three serial passages were carried out by each of three routes: yolk sac, amniotic sac, and chorioallantoic membrane, using embryos of differing ages (Table II). Yolk fluid and sac were harvested from eggs inoculated into the yolk sac after 6 to 7 days of incubation. Those injected by the other two routes were harvested at intervals ranging from 3 to 7 days after inoculation. The amniotic membrane,

TABLE II  
*Methods Employed in Passing Embryonated Eggs*

Route of inoculation	Age of embryos	Original inoculum		Harvest		
		Dilution	Dose	Day	Tissue	Fluid
	days		ml.			
Yolk sac	6-7	Undiluted	0.5	6-7	Yolk sac	Yolk
		$10^{-1}$	0.5	6-7	" "	"
Amniotic sac	11-12	Undiluted	0.2	3-6	Amniotic membrane and embryo	Amniotic
		$10^{-1}$	0.2	3-6	" "	"
Chorioallantoic membrane	11-12	Undiluted	0.2	4-7	Chorioallantoic membrane	Allantoic
		$10^{-1}$	0.2	4-7	" "	"

embryo, and amniotic fluid were taken from amniotically inoculated eggs, but only the chorioallantoic membrane and allantoic fluid were taken from those inoculated on the membrane. Ten to 20 per cent (by weight) suspensions of tissues, made up in the respective fluids harvested, were utilized for passage inocula. Penicillin and streptomycin, 100 units of each per egg, were mixed with the original stool suspension and all passage inocula, cultures of which yielded no growth in sterility broth and thioglycollate medium.

In general the distribution of deaths and the minor anatomical changes observed in this group of eggs resembled those found in the previous group. There was no discernible pattern of deaths or lesions. Tissues and fluids from dead eggs or those which showed lesions, however, were included in each inoculum.

The harvest of the third serial passage was employed to inoculate volunteers. A pool of the yolk sac and amniotic sac harvests was made and each of three volunteers given 4 to 5 ml. of the mixture to drink. Three others each drank an equal amount of the harvest from the chorioallantoic membrane eggs. All six remained symptom-free, and reinoculation with the same pools 4 days later likewise had no effect.

#### DISCUSSION

In the great majority of outbreaks of gastroenteritis which have been investigated by the New York State Department of Health in the last two dec-

ades, the recognized methods of bacteriologic investigation have failed to reveal the etiologic agent (15). This is particularly true of those epidemics in which afebrile attacks are the rule.

Reimann, Price, and Hodges (16) have reported experiments in human volunteers which suggested that the incitant of a clinically similar disease was present in filtered throat washings and fecal suspensions of patients and could be transmitted by the respiratory but not by the alimentary route. Their investigations were carried out without precautions for isolation during the course of a natural epidemic (17). Within a few hours after collection of specimens of stool or throat washing, Mandler filtrates were inoculated by the respiratory or alimentary routes. The inoculations were done on 6 different days so that presumably the inoculum came from at least six different donors. The effects of inocula from each donor were not specifically given, but over half of fifty-three men who inhaled nebulized filtered stool or throat washings developed gastroenteritis. Incubation periods varied between 1 and 21 days. There was no illness among fifteen subjects who swallowed capsules containing a total of 3 ml. of filtered washings or the four who similarly took an equal dose of stool filtrates.

It appears not unlikely that different etiologic agents were responsible for the disease studied by Reimann and his colleagues, and that described in this paper. The variations in epidemiologic and clinical pattern of outbreaks investigated by this Department suggest that gastroenteritis may be caused by more than one unrecognized agent. This might explain the discrepancies between the respective results. Technical factors, however, may be responsible.

Hodges (18) found that penicillin and streptomycin inhibited bacterial growth in embryonated eggs inoculated amniotically with centrifuged unfiltered stool specimens. The present studies confirm this observation; in addition, they demonstrate that it is also true of eggs inoculated by the yolk sac or the chorio-allantoic membrane. The etiologic agent of epidemic gastroenteritis apparently did not multiply in eggs inoculated by these methods, since human volunteers fed tissue extracts and extraembryonic fluids from third passage eggs remained asymptomatic. Possibly the penicillin or streptomycin inactivated the agent. Penicillin inhibits the growth of psittacosis virus in chick embryos (19) for example, although some viruses propagate well in the presence of both antibiotics.

The demonstration that the causative agent of the form of epidemic gastroenteritis here described is present in fecal filtrates and that the disease is readily reproduced in volunteers by oral inoculation should be helpful in orienting further epidemiologic studies and in attempts to establish the agent in experimental animals. Full identification of the agent awaits the successful completion of such studies.

#### SUMMARY

Epidemic gastroenteritis was transmitted to human volunteers by the oral administration of fecal filtrates. The original inocula were obtained from

patients in a natural outbreak which occurred at Marcy State Hospital in the winter of 1946-47. The experimental disease closely resembled that of the donors. The incubation period ranged from 1 to 5 days, with a mean of 3 days. The disease was carried through three generations, in the last two by means of fecal filtrates.

Oral administration of unfiltered throat washings from experimental cases of the disease likewise induced gastroenteritis but subjects who inhaled a portion of the same throat washings remained asymptomatic. Volunteers who inhaled throat washings taken from patients in the epidemic at Marcy State Hospital also failed to develop the disease.

Five volunteers who had previously been inoculated with fecal filtrates were reinoculated with the same material. Gastroenteritis followed in one of the two subjects who had failed to contract the disease the first time. The others remained well.

Embryonated hens' eggs were inoculated with one of the two unfiltered stool suspensions used in the pool which had induced gastroenteritis in each of the three volunteers to whom it was fed. Three sets of eggs were inoculated: one on the chorioallantoic membrane, another into the yolk sac, and a third into the amniotic sac. Three serial passages were carried out by each method at varying time intervals. Penicillin and streptomycin were employed as antibacterial agents. Tissue and extraembryonic fluids from the third passage were non-infective for volunteers.

These experiments were done at the New York State Vocational Institution, West Coxsack, New York. We wish to express our gratitude to J. A. Lyons, Commissioner of Correction; to D. D. Scarborough, Superintendent, J. P. Conboy, Assistant Superintendent, and Dr. A. J. Flood, Senior Physician, of the Vocational Institution, and to their respective staffs, for the complete cooperation and support which made these studies possible.

We are indebted to Dr. G. C. Bower of the Marcy State Hospital, Marcy, New York, for aid in the collection and examination of specimens from patients there, and to Leroy Weaver, Acting Superintendent, and Dr. M. E. Pittman, Senior Physician and Clinical Psychiatrist, of the Elmira Reformatory, Elmira, New York, for their cooperation and assistance.

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# THE DEMONSTRATION OF ONE-STEP GROWTH CURVES OF INFLUENZA VIRUSES THROUGH THE BLOCKING EFFECT OF IRRADIATED VIRUS ON FURTHER INFECTION\*

By WERNER HENLE, M.D., GERTRUDE HENLE, M.D.,  
AND EVELYN B. ROSENBERG

(From The Children's Hospital of Philadelphia (Department of Pediatrics, School of Medicine, University of Pennsylvania), Philadelphia)

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Previous studies (1, 2) have shown that the mechanism of infection of susceptible host cells by influenza virus can be divided into at least four separate phases: (I) the adsorption of the virus onto the host cells (2-4); (II) the development of changes in the host cells which alter their function (decrease or discontinuation of host cell multiplication) (2) and exclude certain other viruses from the cells (interference phenomenon) (5-9)—changes which are possibly concomitant with entrance of the virus into the host cells; (III) increase of the virus in association with the cell; and (IV) release of the newly formed virus from the infected tissue to spread the infection to other susceptible cells.

More is known about the first and second phases than about the third and fourth, especially if one includes the available information on the hemagglutination phenomenon in phase I. The present study has therefore been directed chiefly toward an analysis of the later steps in the infectious process, the intracellular propagation of the virus and its release from the infected cells. Attempts were made to obtain "one-step growth curves"<sup>1</sup> similar to those reported for the propagation of bacterial viruses by Ellis and Delbrück (10) and Delbrück and Luria (11). The technic used by these authors had to be modified for application to the chick embryo-influenza virus system. With these modifications the experiments have revealed that multiplication of the influenza viruses is based apparently on general principles similar to those affecting the propagation of the bacterial viruses.

## *Methods and Materials*

*Seed Culture of Virus.*—The PR8 strain of influenza A and the Lee strain of influenza B were employed in these experiments. The agents were passed by the allantoic route in high

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<sup>1</sup> A one-step growth curve represents a single cycle in the increase in virus after infection of a susceptible host. By means of it one can estimate the yield of virus from a given number of host cells, all of them infected within a period of time purposely limited to allow for the association of the infectious agent with the cells.

dilution ( $10^{-7}$ ) to 10 day old chick embryos by a technic previously described (6). After 48 hours of incubation at 36 to 37°C. the allantoic fluids were collected and stored in small volumes in glass-sealed ampules in a dry-ice cabinet. The seed culture was rapidly thawed immediately before use.

*General Growth Curve Technic.*—Adequate numbers of 11 or 12 day old chick embryos (the older embryos were found to be the more satisfactory) were inoculated with 0.2 ml. of suitably diluted seed culture by the allantoic route (usually  $10^{-4.5}$  for PR8 and  $10^{-6.0}$  for Lee) and returned to the incubator. Further injections, which will be described in the text, were given in 0.5 ml. amounts. Groups of five to six of the inoculated embryos for each experimental series were removed from the incubator at given time intervals and 3 to 4 ml. aliquots of allantoic fluid were withdrawn by needle and syringe from each of the eggs without previous chilling. The blood-free samples were pooled according to groups. The remainder of the allantoic fluids was poured off into a graduated cylinder with precautions to avoid rupture of the amnion or yolk sac. The average volume of allantoic fluid per egg was thus determined. The fluids were kept at 4°C. until the last groups were harvested. The preparations were then assayed for their content of active virus by titration in 10 day old chick embryos. Repetition of titrations of some of the fluids which had been stored at 4°C. for 1 week agreed well with the titers originally obtained.

*Titration of Virus.*—The allantoic fluids were diluted in multiples of ten with brain-heart infusion broth to which 100 units of penicillin and 50 gamma of streptomycin had been added per ml. This precaution was taken since the nature of the experiments did not permit adequate tests for bacteriological sterility prior to the titration. The diluted materials were injected in 0.5 ml. amounts into the allantoic cavity of groups of five normal 10 day old chick embryos. After incubation for 72 hours at 36–37°C. the individual allantoic fluids from these embryos were harvested and tested for their capacity to agglutinate chicken erythrocytes. To 0.4 ml. of fluid, 0.2 ml. of a 1 per cent suspension of thrice washed chicken red cells was added. The test mixture was incubated at 4°C. and read in terms of the pattern formed by the cells at the bottom of the tubes (12). The 50 per cent infectivity end point was determined according to the results of the hemagglutination test by the method of Reed and Muench.

*Irradiation of Virus.*—The infected allantoic fluids were dialyzed for 24 hours in cellophane bags against buffered saline solution of pH 7.0 in the approximate ratio of one part of fluid to twenty parts of saline solution, with repeated stirring of the bath, in order to remove the greater part of the urates (6). The dialyzed fluids were next exposed to ultraviolet light according to a method previously described (6). They were assayed for their interfering and hemagglutinating capacities as also outlined previously (1).

#### EXPERIMENTAL

##### *Growth Curves of Influenza Viruses in Untreated Chick Embryos*

Injection of 1000 to 10,000 ID<sub>50</sub> of influenza virus into the allantoic sac of 11 to 12 day old chick embryos did not lead to complete adsorption of the virus onto the cells lining the allantoic sac, a certain amount of the virus, usually between 10 and 30 per cent, remaining free in the allantoic fluid. With the establishment of equilibrium in  $\frac{1}{2}$  to 1 hour, the residual free amount of virus remained constant for a variable number of hours, depending on the strain of virus used. In the case of the PR8 strain of influenza A, 6 hours elapsed after injection, and with the Lee strain of influenza B, 9 to 10 hours before the concentration of virus in the allantoic fluid commenced to rise.



In some experiments the increase in titer during the next few hours was quite rapid, then lessened and rose again steeply later, suggesting a certain periodicity in virus production. In other instances the quantities of active virus increased only very slowly. Figs. 1 and 2 summarize the results of several experiments each with the PR8 and Lee strains. The mean values found in the allantoic fluids at various hours after infection have been linked by solid lines.

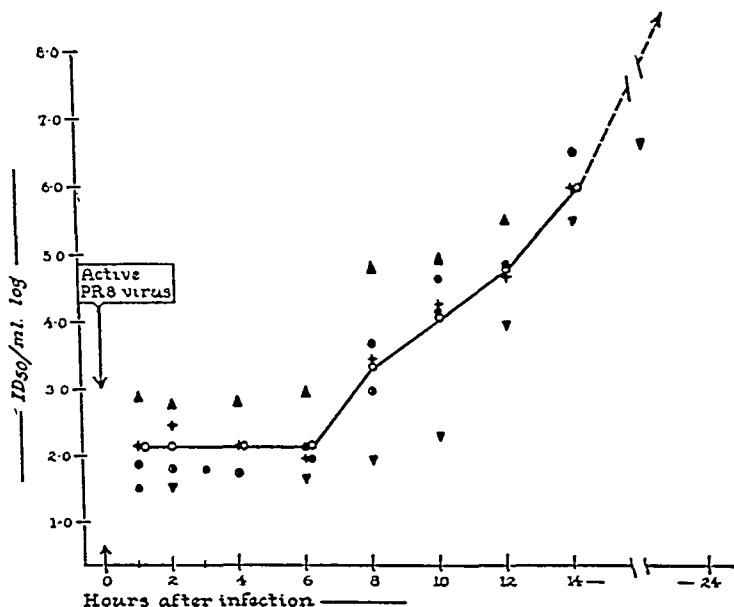


FIG. 1. Increase in the PR8 strain of influenza A virus in chick embryos (five experiments). The mean titers are indicated by the open circles.

These data suggested that the virus increased in association with the cells for about 6 hours in the case of the PR8 strain, and for about 9 hours in the case of the Lee strain before the newly formed agents were released from the injected cells. Thereafter the findings became quite irregular. This variability was presumed to be due to a varying degree of additional adsorption of freshly released virus onto susceptible cells, and hence the apparent titer was not considered an indication of the actual amount of virus produced. This conception was borne out by the experiments described in the next section.

#### *The Effect of Heterologous Irradiated Virus on the Growth Curve of Influenza Virus*

The variations just mentioned have been largely excluded by an adaptation of the "one-step growth technic" already mentioned, which had been de-

veloped for the study of bacterial viruses (10, 11). By this technic bacteria are exposed for a brief period to sufficient bacteriophage to infect most of them with a single particle. Further infection is then checked by dilution. In the supernate of centrifuged aliquots of the infected bacterial suspensions the amount of free virus is determined by the plaque method. The suspension is then incubated and aliquots are tested at intervals for the "infectious centers" present. For a certain fixed period, depending on the bacteriophage used, the number of plaques remains constant. During this constant period the virus is

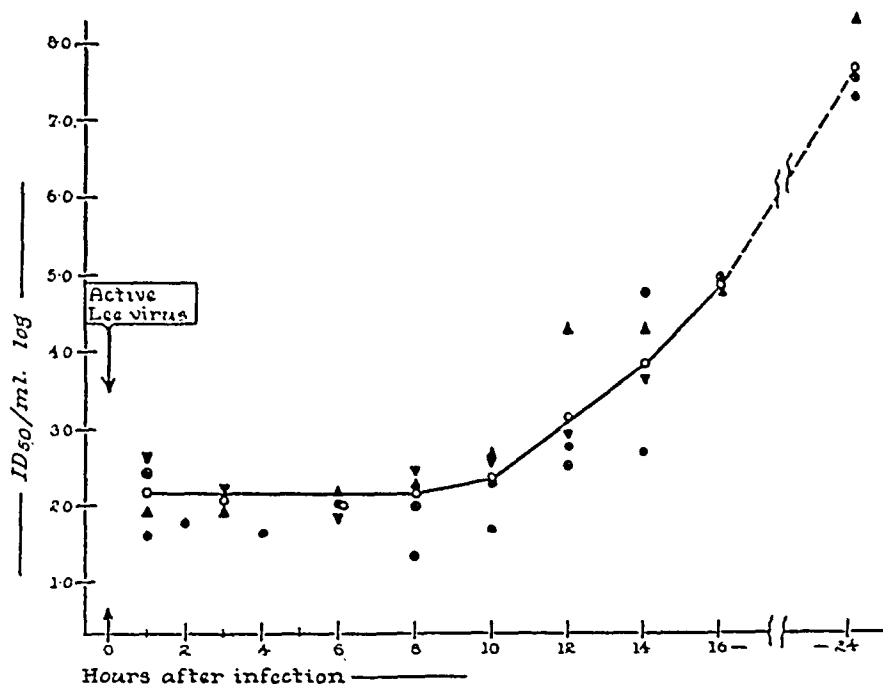


FIG. 2. Increase in the Lee strain of influenza B virus in chick embryos (four experiments). The mean titers are indicated by the open circles.

multiplying within the host. Then there is a sudden rapid increase in the amount of detectable virus, followed by a new plateau. From the data obtained, it is possible to estimate the yield of virus per bacterium.

This technic obviously could not be applied without modifications to the chick embryo-influenza virus system. The following procedure was adopted:—

To obtain "single infection" of cells small doses of active virus were injected at the onset of the experiments; *i.e.*, usually 1000 to 10,000 ID<sub>50</sub>. Then, to interrupt the process of adsorption and to prevent further infection of susceptible cells a second injection was given consisting of 0.5 ml. of allantoic fluid containing irradiated *heterologous* virus. This was sufficient to produce the interference phenomenon in all remaining susceptible cells (6) and to reduce, if not prevent, further adsorption of active virus from the seed culture as well as from the yield. The allantoic fluids of

groups of five to six eggs thus treated were harvested at various time intervals and the content of active virus was assayed by titration in 10 day old chick embryos.

The assumption that the relatively large amounts of irradiated heterologous virus would decrease or prevent the adsorption of the relatively low concentrations of newly formed virus upon release from the infected cells was based on experiments reported in previous publications (1, 2, 6). These earlier data were obtained mainly by using the hemagglutination reaction, which, however, permits detecting the adsorption of only fairly large amounts of virus. Some direct titrations of infectivity for chick embryos were required, therefore, to determine the adsorption of relatively small quantities of virus. Accordingly, groups of 11 day old chick embryos were injected with either 0.5 ml. of irradiated preparations of virus, or with normal allantoic fluid. Three hours later, a second injection of about  $10^5$  to  $10^6$  ID<sub>50</sub> of active homologous or heterologous virus was given. The allantoic fluids of these eggs were collected after further incubation of 2 to 3 hours and pooled according to groups. The infective titers for chick embryos of these fluids multiplied by the volume of allantoic fluid collected represented the total recovery. A comparison of this quantity with the total amount of virus injected revealed no significant decrease of virus if the eggs had been injected previously with irradiated virus, in contrast to the markedly lower recovery of virus in those groups which had received normal allantoic fluid prior to injection.

Examples of the results of the procedure are shown in Table I. They confirm the previous finding that there is little, if any, adsorption of active virus in the allantoic sac of eggs previously injected with large amounts of the irradiated agents.

Using the technic as described, "one-step growth curves" were obtained which resembled closely those of bacterial viruses. As can be seen in Figs. 3 and 4, the content of active virus in the allantoic fluids remained constant for 6 to 9 hours, respectively, depending on whether the PR8 or Lee strains were studied. This amount represented non-adsorbed virus from the inoculum. Thereafter, the concentration of active virus rose steeply for 2 hours in the case of the PR8, and for 2 to 3 hours in the case of the Lee strain. From then on the active virus titer remained on this higher plateau up to the 24th hour after infection, when the experiments were terminated. The figures show the data of five separate experiments each, with closely similar concentrations of active seed virus. The means for the various determinations are connected by the solid lines. A comparison of Figs. 1 and 3, and Figs. 2 and 4, gives emphasis to the supposition that in the absence of blocking by heterologous irradiated virus, some of the newly formed active virus is adsorbed onto the allantoic sac upon its release from the cells.

Varying the concentration of active virus in the inoculum over a wide range did not result in significant alterations in the "one-step growth curves." The constant period for the PR8 strain remained 6 hours, as seen in Fig. 5. Similar results were obtained with the Lee strain except that in this case the

TABLE I  
*Prevention of Adsorption of Active Virus by the Injection of Irradiated Virus Prior to Infection*

1st Injection	2nd Injection		Result of harvest		
Irradiated allantoic fluid	Active virus	ID <sub>50</sub>	Allantoic fluid	ID <sub>50</sub> /ml.	Total ID <sub>50</sub> recovered
			ml.		
PR8.....	PR8	10 <sup>5.64</sup>	7.6	10 <sup>4.43</sup>	10 <sup>5.21</sup>
Lee.....	PR8	10 <sup>5.64</sup>	9.2	10 <sup>4.47</sup>	10 <sup>5.13</sup>
Normal.....	PR8	10 <sup>5.64</sup>	7.7	10 <sup>3.34</sup>	10 <sup>4.73</sup>
PR8.....	Lee	10 <sup>5.40</sup>	7.0	10 <sup>4.47</sup>	10 <sup>5.23</sup>
Lee.....	Lee	10 <sup>5.40</sup>	6.8	10 <sup>4.53</sup>	10 <sup>5.28</sup>
Normal.....	Lee	10 <sup>5.40</sup>	7.3	10 <sup>3.30</sup>	10 <sup>4.17</sup>

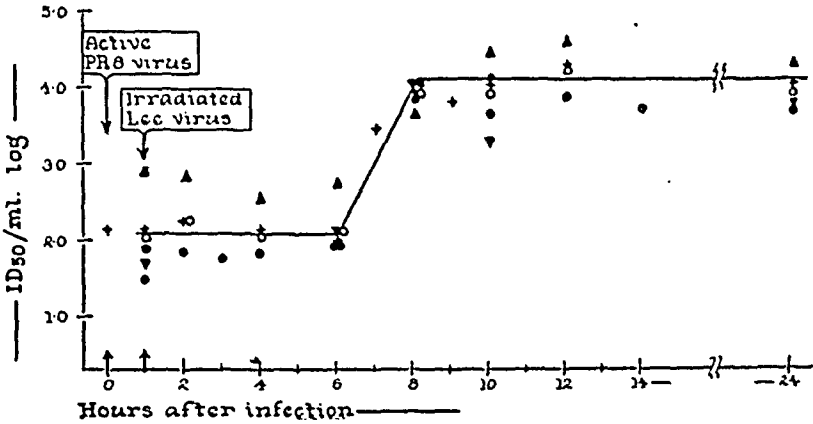


FIG. 3. One-step growth curve of the PR8 strain of influenza A (five experiments). The mean titers are indicated by the open circles.

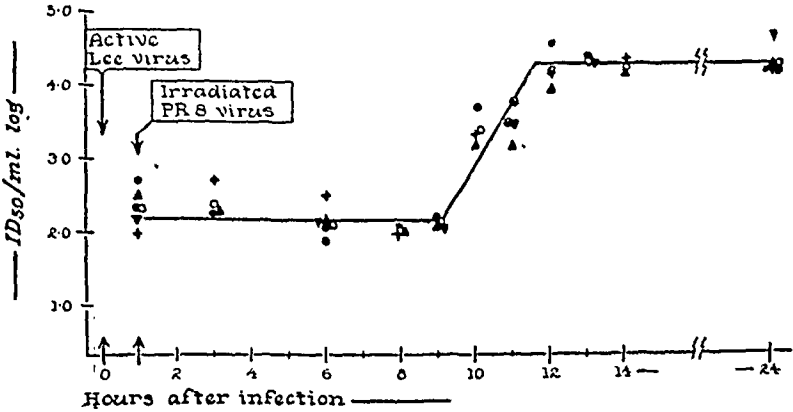


FIG. 4. One-step growth curve of the Lee strain of influenza B (five experiments). The mean titers are indicated by the open circles.

constant period was regularly 9 hours. When  $10^{1.9}$  ID<sub>50</sub> of active PR8 virus were injected the titrations of the harvests had to be begun with undiluted allantoic fluids. These, however, contained sufficient concentrations of non-adsorbed irradiated heterologous virus (Lee) to cause interference in the low dilutions of the titrations. It was necessary, therefore, to add to the dilutions of the harvested allantoic fluids rabbit anti-Lee serum to prevent the interfering effect. The serum did not apparently influence the increase in quantity of the active PR8 virus.

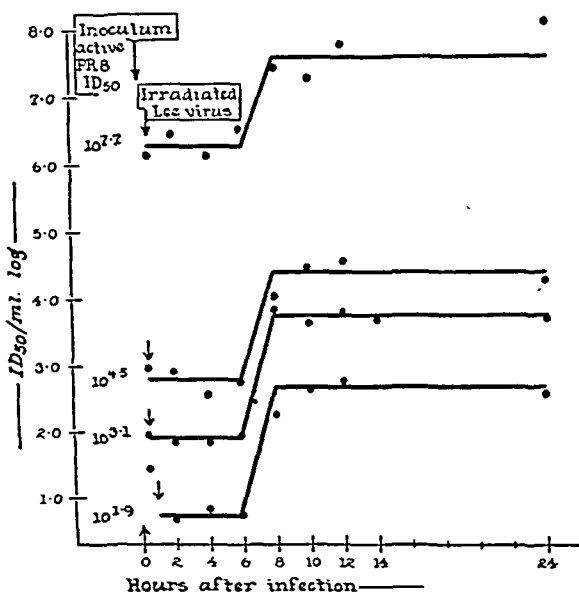


FIG. 5. One-step growth curves following injection of various concentrations of active virus.

The "one-step growth curves" were practically alike when either the usual amounts of heterologous virus (undiluted allantoic fluid) or five times that amount of virus (concentrated by high-speed centrifugation) were injected one-half hour after infection. The individual end points of the various titrations did not differ significantly except for the slight variations inherent in the reaction. Such experiments furnished additional evidence that the amount of heterologous irradiated virus injected in the various tests described in this paper was sufficient to prevent extensive adsorption of the freshly released virus. Upon five-fold dilution, on the other hand, the amount of heterologous irradiated virus was insufficient to prevent additional adsorption.

In one experiment, the interval between infection and administration of the irradiated virus was varied and its effect on the "one-step growth curve"

studied. The embryos were infected with active PR8 virus and received irradiated Lee virus, 1, 3, and 6 hours after infection. The "one-step growth curves" for the 1 and 3 hour series were practically alike. However, after 6

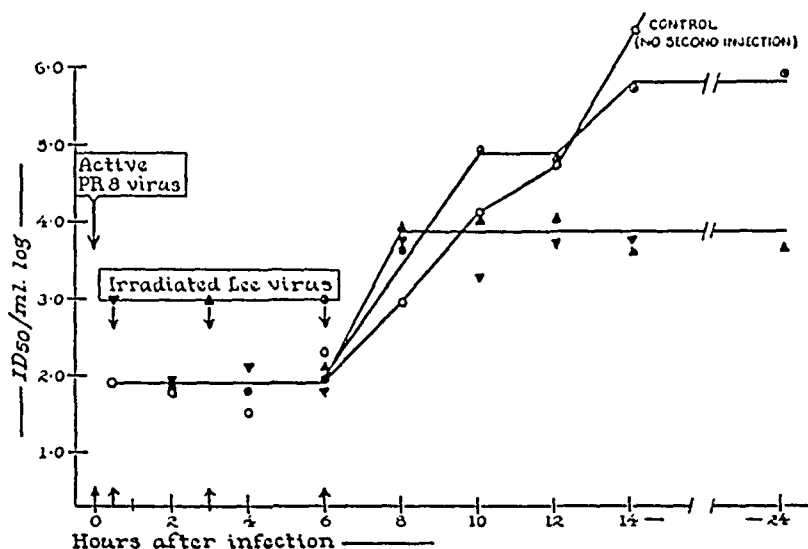


FIG. 6. Growth curves following injection of irradiated heterologous virus at various hours after infection.

hours, the heterologous virus was given at the beginning of the first step and consequently some further cells became infected during the period before the irradiated virus was injected. Thus, the plateau finally reached was approximately 100-fold higher than that obtained in the 1 and 3 hour groups. This experiment is summarized in Fig. 6.

From the data presented in Figs. 3 and 4, attempts were made to calculate the quantities of virus produced.

The calculations were necessarily hampered by the inaccuracies inherent in the methods of virus assay and, to a lesser extent, by the technical difficulties in the quantitative collection of allantoic fluid. However, when the various experiments were combined which involved comparable amounts of virus as inoculum, although from different seed cultures (PR8) or similar dilutions of the same seed cultures (Lee), the calculations gave some information at least as to the order of magnitude of the production of virus in the cells of the allantoic sac. The calculations shown in Table II summarize five experiments each with the two respective strains. With each experiment the seed was titrated by the injection of tenfold dilutions into eight to ten eggs each. The amount injected in the experiments was calculated from the results of these titrations. The free virus in the allantoic fluids during the constant period represented virus from the inoculum which had not been adsorbed. This amount has been calculated by multiplying the mean ID<sub>50</sub> per ml. during the first 6 hours in the case of the PR8 virus, and during the first 9 hours in the case of the Lee strain, with the average volume of allantoic fluid harvested. This value sub-

tracted from the inoculum constituted the amount of virus adsorbed. The average total amount of virus after the step (10 to 24 hours after infection with PR8, and 12 to 24 hours after administration of active Lee virus) minus the active virus found in the allantoic fluid during the constant period represented the amount of virus produced. This value divided by the  $ID_{50}$  adsorbed corresponds to the number of  $ID_{50}$  produced per one  $ID_{50}$  adsorbed. As can be seen in Table II, these values fell between 45 and 79, with an average of 63 in the case of PR8 virus, and between 25 and 55, with an average of 36, for the Lee virus.

In view of the variations already described, the apparent difference in yield of PR8 and Lee viruses cannot be taken as absolute and it is possible that a

TABLE II  
*Calculation of Yield of Virus (Five Experiments Each)*

	PR8 ( $ID_{50}$ )		Lee ( $ID_{50}$ )	
	Average	Range	Average	Range
(1) Inoculum.....	1540	1200- 2000	5250	4000- 7900
(2) Recovered during constant period.....	600	300- 1000	1100	1000- 1250
(3) Adsorbed; (1)-(2).....	940	590- 1500	4150	3000- 6800
(4) Recovered after the step..	59,500	32,500-96,000	149,000	106,000-230,000
(5) Total produced; (4)-(2)...	58,900	32,010-95,500	147,900	105,000-228,900
(6) $\frac{ID_{50} \text{ produced (5)}}{ID_{50} \text{ adsorbed (3)}}$	63	45- 79	36	25- 55

larger series of tests would have lessened these differences. As pointed out, the calculations should be taken only as an indication of the order of magnitude of virus production.

#### *Depression of Yield of Virus by the Homologous Irradiated Agent*

The earlier experiments were conducted with heterologous irradiated virus because it was anticipated that under certain conditions the interfering effect of the non-adsorbed irradiated virus would have to be neutralized by immune serum, as discussed above. This turned out to be a more fortunate choice than anticipated because it was found, subsequently, that homologous irradiated virus exerted a marked depressing effect on the step size in the growth curve experiments. As can be seen in Figs. 7 and 8, the step in the PR8 tests (two experiments) was strikingly delayed and decreased in size. In the four Lee experiments no step occurred in two, and only a slight rise in virus titer in the others. These experiments were performed simultaneously with tests in which heterologous irradiated virus was utilized, and the fluids giving the depressor effect were tested against heterologous active virus. Both tests gave the usual one-step curves. Thus it is apparent that homologous irradiated

virus may have a profound effect on the production of active virus in cells which had been infected prior to the administration of the irradiated agent, or

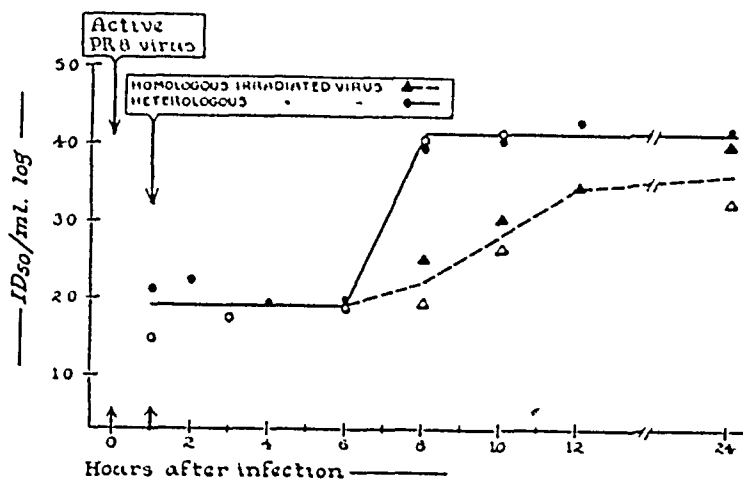


FIG. 7. Depression of the yield of virus by homologous irradiated virus (two PR8 experiments).

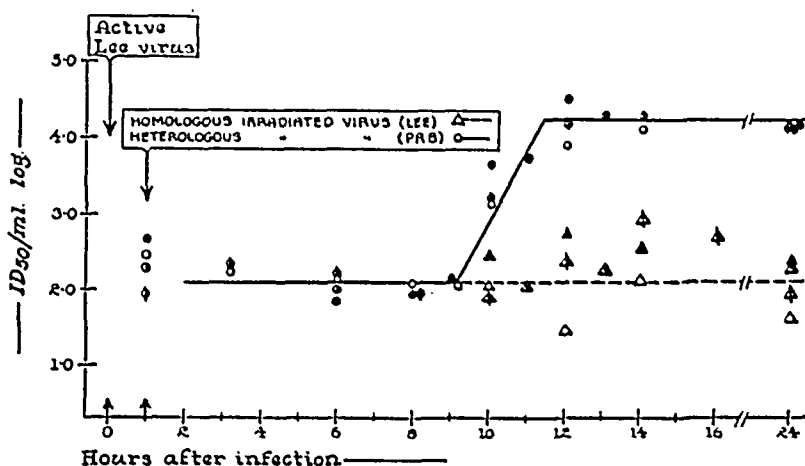


FIG. 8. Depression of the yield of virus by homologous irradiated virus (four Lee experiments). The results of tests utilizing the homologous irradiated virus are indicated by the various triangles; of those utilizing the heterologous irradiated virus by the various circles.

its release from such elements. This effect is not shared by heterologous irradiated virus.

The degree of depression in the yield of virus by homologous irradiated virus decreased gradually with the increase in the interval between infection and administration of the inactivated agent. Fig. 9 shows the results of an experiment with the Lee strain. It is to be noted that the size of the step increased gradually, but even when injected 4 hours after infection homologous irradiated



virus still depressed the yield to some extent. When given after 6 hours a one-step growth curve was obtained similar to that noted following injection of heterologous irradiated virus 1 hour after infection.

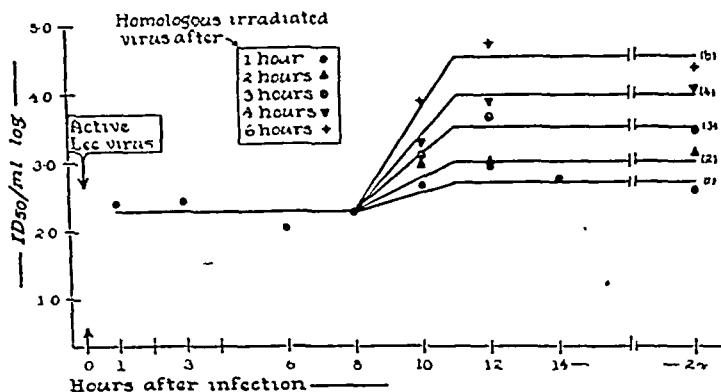


FIG. 9. Depression of the yield of active virus by homologous irradiated virus injected at various hours after infection.

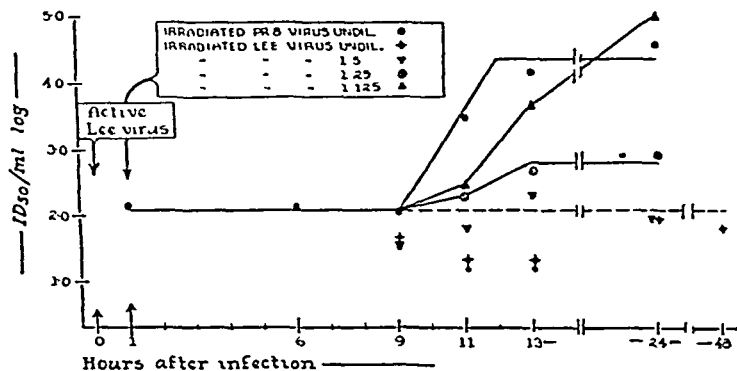


FIG. 10. Depression of the yield of active virus by decreasing amounts of homologous irradiated virus.

The inhibitory effect of the homologous inactivated agent still was very marked when the irradiated allantoic fluid was diluted fivefold before injection. Upon further dilution in fivefold steps the effect decreased, as shown in Fig. 10 and the growth curve finally became comparable to those obtained in untreated chick embryos (Fig. 2.).

The agent responsible for the decrease in yield of virus was sedimentable, together with that responsible for the hemagglutinating property, by centrifugation of the preparation at 20,000 R.P.M. for 20 minutes. The supernatant fluid was ineffective in that it failed to depress the yield of active virus and the

growth curves resembled those shown in Figs. 1 and 2, obtained with infected eggs which received no further treatment. Irradiation of the homologous virus for prolonged periods of time likewise eliminated the inhibitory effect. In this case the interfering capacity had been destroyed by the irradiation but the hemagglutinating capacity was left partially intact (1).

It has not been possible as yet to determine whether the inhibitory effect is consequent on reduction of the amount of increase of the virus, associated with cells, or whether it is due to prevention or delay in its liberation. However, an experiment with the Lee strain carried over 48 hours did not disclose a delayed rise in virus titer. Longer intervals have not been tested. Attempts to determine the amounts of active virus in suspensions of carefully washed allantoic sacs harvested at various intervals after infection did not meet with success: only irregular results were obtained.

### DISCUSSION

In a previous publication (2) it has been pointed out that there exist striking similarities between certain bacterial viruses and the agents of epidemic influenza in their host-virus interrelationships. These similarities were apparent in the conditions of adsorption of the viruses onto the host cells, the various aspects of the interference phenomenon between the inactivated agents and the active viruses, and the effects of the agents on the functions involving the propagation and metabolism of the host cells. The present study extends this agreement to the general appearance of the growth curves given by these agents. After entrance of the virus into the host bacterium, it multiplied within for a definite period of time which was found to be characteristic for each strain of bacterial virus studied (11). This period of propagation in association with the cell seems, likewise, characteristically different for the influenza viruses. In the case of the PR8 strain, it lasts about 6 hours, and in that of the Lee virus, about 9 hours. A few preliminary tests with other strains of influenza virus indicated that they may require shorter, or intermediate, periods of time for intracellular multiplication.

No information is available as to what happens within the cells during this latent period. Attempts to demonstrate a rise in the content of virus in suspensions of emulsified allantoic sacs harvested during the latent period have failed to produce reliable results, partly on account of technical difficulties such as incomplete liberation of the virus from the tissue and adsorption of the virus onto tissue debris, red cells, etc., partly, perhaps, for biological reasons; *i.e.*, the virus may pass through developmental stages in which it lacks infectivity as yet, and therefore cannot be demonstrated by the available technics.

At the end of the period of propagation the newly developed virus is released from the infected cells. This period is relatively short in the case of the bacterial viruses (11), which is in keeping with their faster growth cycle. In the

influenza tests, this period lasts for 2 to 3 hours until all the virus to be released from the infected cells has come away into the allantoic fluid. The amount of bacterial virus produced under standard conditions of culture of the host cells is again characteristic of the individual strain of virus (11). It is not possible, as yet, to make a similar statement in regard to the influenza viruses, since the technics of virus assay are not sensitive enough. The use of five chick embryos per dilution in infectivity titrations leads to results which may be accurate within  $\log 0.6$  (13), and the accuracy of such titrations can be increased by the use of greater numbers of eggs per dilution or by performing a greater number of parallel titrations and determinations of the mean. The present experiments gave practically the same results as this latter method since all titrations of the allantoic fluids obtained during the constant period, and again of those collected after the plateau was reached, could be combined for the final evaluation. In addition the Lee experiments were all conducted with the same seed culture using the same dilution, so that the results of the five tests could be combined. In consequence the calculations gain somewhat in accuracy. However, all that can be said at present is that the number of  $ID_{50}$  of influenza virus produced per one  $ID_{50}$  adsorbed is of the order of 50. It is possible that the yield of PR8 virus exceeds somewhat that of the Lee strain. The number of virus particles constituting one  $ID_{50}$  has not been definitely determined. According to Friedewald and Pickels (14), who used centrifugally purified preparations, it amounts to about ten virus particles. In view of the fact that only part of these will be adsorbed, that some of them may have been inactivated by the methods of concentration, that possibly not all sedimentable material was virus, and that the experimenter has to do with the amount of virus infecting only 50 per cent of the eggs, the suggestion seems not amiss that one particle of influenza virus may cause infection of the chick embryo.

It appears most likely that the influenza virus freshly yielded into the allantoic fluid is released by the destruction of the entodermal cells of the allantoic sac, just as happens when bacteria are lysed by bacteriophage. It has been observed in infections with influenza virus that the epithelial lining of certain sections of the respiratory tract of ferrets and mice is largely destroyed (15-17). No studies of the lesions encountered in the allantoic sac of the chick embryo have been published except for a brief account by Burnet (18) who found only minor changes in the entodermal layer of the allantoic sac, save for damage of occasional individual cells or groups of cells. A few attempts to study the histological appearance of some of the allantoic sacs from the growth curve experiments have not been revealing. The number of infected cells was presumably very small since the infecting virus dose was limited to 1000 to 10,000  $ID_{50}$  in most of the experiments, and further infection was prevented by the irradiated virus. However, when the allantoic sacs of chick embryos were examined 48 or 72 hours after injection of active virus only, that is to say at a

time when the titer of virus in the allantoic fluid had reached its peak, the entodermal epithelium appeared largely destroyed or severely damaged. It remains to be seen whether this destruction of the entodermal cells occurs concurrently with the appearance of fresh virus in the allantoic fluid, or whether the virus comes away as a result of some other process and the host cells are destroyed later. This question gains in importance in the light of the observations made on the depression of yield of active virus by the homologous irradiated agent, to be discussed further on.

An inhibiting effect has been demonstrated by Delbrück (19) in certain interference experiments with two distinct, active, bacterial viruses. The result of exclusion of the heterologous agent was a decrease in the yield of virus multiplying within the host cells, whereas exclusion of homologous virus was apparently without inhibitory effect. Addition of antiserum against the heterologous agent largely prevented the inhibition. The amount of suppression decreased also with the increase in the interval between infection of the bacteria and addition of the heterologous virus. This inhibitory effect in bacterial virus infections has many similarities with that now observed in the influenza system except that in the latter case the homologous virus produced the inhibition, not the heterologous, and furthermore the preparations had been inactivated by irradiation with ultraviolet light. Because homologous virus was the effective agent, it was impossible to test for neutralization of the inhibitory influence with specific immune serum, since residual free antibodies would have affected freshly produced active virus upon its release from the entoderm into the allantoic fluid. In the one-step growth experiments based upon the use of heterologous irradiated virus, injection of the corresponding antiserum did not increase the size of the step, indicating that the heterologous agent had no inhibitory effect.

The inhibition is presumably mediated by the virus particle but its mechanism is unknown. It may hinder the multiplication of the virus or prevent its release from the cells. It should be recalled in this relation that the preliminary injection of irradiated virus prevents infection by homologous and heterologous virus equally well (6). Conceivably, when introduced after infection, the homologous irradiated virus may still enter the cell and interfere with some stage of the development of the active virus, whereas the heterologous agent is unable to follow this course because a stage in the infectious cycle has been reached in which the heterologous agent cannot participate.

#### SUMMARY

After allantoic injection of chick embryos with a known amount of influenza virus, the process of adsorption of the agent onto host cells and infection of them can be interrupted at a given time by the administration of large quantities of heterologous virus inactivated by irradiation. A sudden great increase

in the amount of free virus in the allantoic fluid occurring after 6 hours in the case of the PR8 strain, and 9 hours in that of the Lee strain, indicates that the untreated virus associated with the host cells has multiplied. The length of the period preliminary to this increase remains the same even though the concentration of the original inoculum is varied over a wide range. Since administration of the irradiated virus leaves no susceptible host cells, because of the interference phenomenon, and further adsorption of active virus is minimized or entirely prevented, practically the entire new increment of virus can be found in the allantoic fluid and assayed; for every  $ID_{50}$  adsorbed about 50  $ID_{50}$  are released. Homologous irradiated virus, on the other hand, when injected after infection of the allantoic sac, reduces the yield of virus to a more or less considerable extent. Some inhibitory effect can still be observed when the homologous irradiated virus is given several hours after infection. This effect is linked to the virus particle and destroyed by prolonged irradiation.

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# STEPWISE INTRATYPE TRANSFORMATION OF PNEUMOCOCCUS FROM R TO S BY WAY OF A VARIANT INTERMEDIATE IN CAPSULAR POLYSACCHARIDE PRODUCTION

By COLIN M. MACLEOD, M.D., AND MARJORIE R. KRAUSS

(From the Department of Bacteriology, New York University College of Medicine, New York)

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## INTRODUCTION

Previous studies of pneumococcal transformation have been concerned in the main with the conversion of R organisms derived from one specific type to S organisms of heterologous type. The present investigations are concerned with intratype conversion or transformation of pneumococci. A stable variant of pneumococcus Type II, intermediate between the classical R and S forms, has been isolated. It has been demonstrated that the variant produces a certain amount of specific capsular polysaccharide (SSSII) although it does not possess a demonstrable capsule nor does it form colonies with a smooth surface similar to those of S organisms. The gene-like material controlling the formation of SSSII has been separated from the intermediate variant and transferred *in vitro* to an R strain of pneumococcus which thereby assumes certain characteristics of the intermediate. The transformed R strain retains its rough colonial form, produces SSSII, but has no demonstrable capsule. In addition, the intermediate has been converted *in vivo* to a fully encapsulated strain of pneumococcus Type II.

A variety of variants intermediate between the R and S forms of pneumococci have been described before. Blake and Trask (1) in 1923 noted the presence of intermediates derived from pneumococcus Type I and described 5 of these in detail 10 years later (2). Of the 5 intermediate variants derived from Type I by serial cultivation in broth containing Type I antipneumococcal serum, 2 were relatively stable on cultivation in broth, but both of these reverted to fully encapsulated Type I S on mouse passage. Blake and Trask (2) likewise described an intermediate originating from pneumococcus Type II. The occurrence of intermediate variants has also been reported by Dawson (3), Neufeld and Levinthal (4), Klumpen (5), Paul (6), and by others.

It has been customary in the past to consider that cultivation of S pneumococci in media containing homologous anticapsular antibodies suppresses in some manner the polysaccharide-synthesizing mechanism, and that con-

versely, the reversion of R pneumococci to the homologous S form on cultivation in anti-R serum involves reactivation of capsular synthesis. In the light of modern knowledge it seems more probable that R and intermediate mutants occur in cultures of pneumococci of various specific types and that these mutants have a selective advantage when the predominantly S cultures are grown in the presence of anti-S serum. Similarly, some, but not all, cultures of R pneumococci contain a small number of S and intermediate forms or else occasionally throw off S mutants which are selected when cultivated in anti-R serum or injected into mice. That the degradation from S to R may involve a stepwise loss of characters is indicated by earlier reports (1-6). The present studies describe a specifically directed, stepwise restoration from R to intermediate and then to the fully encapsulated S form.

### *Materials and Methods*

*1. Preparation of Transforming Extracts.*—In previous methods for the preparation of transforming extracts (7) the bacterial cells obtained by centrifuging young broth cultures of encapsulated pneumococci have served as the starting material. Very large amounts of broth culture must be used to obtain even a modest amount of active transforming extract. In the present method advantage was taken of the mass culture technique in which an excess of glucose is added to the medium and the acid produced during the course of growth is neutralized intermittently with NaOH. A much greater yield of bacterial cells per volume of medium can be obtained under these circumstances. In addition, by observation of the rate of glucose utilization, the transforming material can be harvested from bacteria in the more active phase of growth.

One liter of nutrient broth prepared from fresh beef heart infusion and containing 1 per cent neopeptone was seeded with 15 cc. of the supernatant of an 18 hour rabbit blood broth culture of pneumococci. After incubation at 37°C. for approximately 16 hours, the organisms were all, or virtually all, Gram-positive. Glucose was then added to a final concentration of 0.5 per cent and 1 cc. of 0.5 per cent phenol red in 95 per cent alcohol was added as indicator of acid production (0.0005 per cent phenol red). Following the addition of glucose, incubation was continued for a period of 3 to 6 hours depending on the growth characteristics of the strain of pneumococcus. During the last 15 minutes the culture was grown in the presence of 0.001 molar sodium citrate to inhibit destruction of the transforming principle (8). Growth was stopped when the slope of the curve for the addition of alkali began to fall off. At this time sodium citrate was added to a concentration of 0.1 M. Sodium desoxycholate was then added to a concentration of 0.05 per cent. The broth culture, which had been creamy with cells, cleared within approximately 3 minutes and became only moderately opalescent and somewhat viscous depending on the type of pneumococcus that was lysed.

An attempt to grow pneumococci in the presence of a small amount of sodium citrate (0.0001 M) was made but abandoned because the growth rate was reduced to about one-third that obtained in citrate-free medium.

To the desoxycholate-lysed culture, one volume of alcohol was added with shaking whereupon a white fibrous precipitate separated out. The mixture was centrifuged and the supernatant discarded. The precipitate was dissolved in 100 cc. of 0.1 M sodium chloride solution containing 0.1 M sodium citrate. Protein was removed by repeated shaking with a mixture of 20 cc. of chloroform and 5 cc. of octyl alcohol in a shaking machine. After the third chloroform treatment only a very slight film of protein-chloroform gel was visible at the inter-



face following centrifugation. The transforming principle was then precipitated with two volumes of alcohol. The active principle was partially purified by dissolving the precipitate in saline and reprecipitating with two volumes of alcohol. After the second precipitation the active fraction appeared as a dense white fibrous mass.

A difficulty encountered through addition of 0.1 M sodium citrate is the precipitation of large amounts of crystalline sodium citrate when one volume of alcohol is added and the mixture allowed to stand overnight in the cold. The active fraction becomes entrapped in the crystalline deposit. This difficulty can be avoided by completing the separation and partial purification of the desoxyribonucleic acid fraction during the course of a single day, and avoiding storage in the ice box during these stages.

2. *Transformation Reactions in Vitro*.—A method previously described (7) was used with minor modifications. One drop of a  $10^{-4}$  dilution of an 18 hour blood broth culture of the R or intermediate variant of Type II pneumococcus was seeded into charcoal-absorbed broth containing an appropriate amount of transforming principle and 10 per cent anti-R pleural fluid which had been heated previously at 60° for 30 minutes. The volume of broth, chest fluid, and transforming principle was 2 cc. in all. After 24 hours' incubation, transfers were streaked on the surface of blood agar plates and subcultures of the supernatant were made in 10 per cent anti-R serum broth with or without further addition of transforming principle. The supernatants of all cultures were examined carefully for turbidity as an indication of the occurrence of transformation. In all instances the experiments were set up in duplicate.

3. *Transformation Reactions in Vivo*.—The technique followed was that described originally by Griffith (9) in which mice are injected subcutaneously with small amounts of living R forms mixed with large amounts of heat-killed smooth organisms.

To obtain a mass growth of smooth organisms, a liter of neopeptone infusion broth was seeded with smooth pneumococci and incubated at 37°C. overnight. The next morning 10 gm. of glucose was added and the culture was incubated for 6½ hours with intermittent neutralization. During the last half-hour the culture grew in the presence of 0.001 M sodium citrate. The culture was then centrifuged in the presence of approximately 0.1 M sodium citrate and the pneumococci were resuspended in 0.1 M sodium citrate-normal saline solution. The organisms were killed by heating at 65°C. for 30 minutes in a water bath. Pyrex tubes were half filled with the bacterial suspension. The mouth of each tube was flamed thoroughly and the tubes immersed in the water bath to the lip. The suspension of heat-killed organisms was streaked on blood agar plates, inoculated into broth and injected into mice. There was no evidence of living pneumococci from any of these tests.

White mice of the CFW strain were injected subcutaneously in the lower abdominal region with 0.1 cc. of an 18 hour blood broth culture of R pneumococci and the suspension of heat-killed S organisms in an amount sufficient to make the mixture as turbid as possible and still allow injection through a No. 25 hypodermic needle. A total volume of 0.5 cc. was injected into each mouse. Upon death of the mice, organisms obtained from peritoneal washings and cultures of heart blood were examined for capsular swelling with type-specific serum and the heart blood was streaked on rabbit blood agar plates for subsequent study.

4. *Strains of Pneumococcus*.—D39/S: a fully encapsulated strain of pneumococcus Type II.

D39/R19: a rough strain obtained by growing the Type II S strain D39/S for 19 serial transfers in Type II antipneumococcal rabbit serum.

D39/Int53: a strain intermediate between the classical R and S forms with respect to SSSII formation, derived from the rough strain D39/R19 by selection.

R36NC: colonial variant of the rough strain R36 which was used in studies by Avery, MacLeod, and McCarty (7). R36 was derived originally from Type II strain D39S.

A/66: a fully encapsulated strain of pneumococcus Type III also used in previous studies (7).

5. *Anti-R Serum*.—Sterile pleural fluids containing R agglutinins were obtained from patients suffering from streptococcal pneumonia and tuberculosis respectively. The pleural fluids were clarified by centrifugation and heated at 60° for 30 minutes before being used in the tests.

#### EXPERIMENTAL

*Isolation of Intermediate Variant D39/Int53*.—Dawson (10) observed that in broth cultures seeded with different amounts of R and S pneumococci the same relative proportions of R and S forms were maintained for several transfers. In other words, when R and S pneumococci are grown together in broth culture, neither form appears to have a selective advantage over the other. This observation of Dawson's has been confirmed in the present study. However, when an anti-R serum is added to the medium, S pneumococci appear to have a selective advantage over R although it is possible that some R cells under these circumstances might be transformed to S cells because of the transforming principle present in the S organisms.

When a mixed culture containing a large seeding of R organisms and a comparatively small number of S organisms is grown in broth it is a matter of chance whether any S forms will be carried along when transfers are made using only a loopful of whole culture. On the other hand, if such a mixed culture is grown in the presence of anti-R serum, the R organisms are agglutinated in the bottom of the tube and unagglutinated organisms grow throughout the supernatant. Thus when a mixed culture is grown in the presence of anti-R serum and the supernatant only transferred, the unagglutinated organisms will be recovered even though they are present in comparatively small number in the original inoculum.

When the cells from 10 cc. of whole broth culture of the rough strain D39/R19 and  $1 \times 10^{-8}$  cc. of culture of the smooth strain D39/S were inoculated together into 4 cc. of 10 per cent anti-R serum broth, S pneumococci were demonstrated on plating the first subculture even though the ratio of R to S in the original inoculum was  $10^9:1$ . The transfer was made by centrifuging the 24 hour mixed culture for 5 minutes at low speed to pack the R cells and transferring the entire supernatant to 4 cc. of 10 per cent anti-R serum broth. The original mixed culture was grown in a long narrow tube, 12.5 cm. by 8 mm. This afforded a tall column of medium through which non-agglutinated organisms could grow and permitted easy removal of the supernatant. Tubes of this sort are not a necessary prerequisite but make it easier to obtain the supernatant relatively free of R cells. The efficiency of this method of selection is indicated by the ease with which 1-3 S cells could be detected when inoculated along with  $10^9$  R cells.

By using this technique it was found that when the rough strain D39/R19 was grown in 10 per cent anti-R serum broth and all or only a loopful of supernatant was used in making transfers to serum broth, intermediate variants

could be selected readily in three subcultures. These variants, although forming colonies with a rough surface, were not agglutinated when grown in 10 per cent anti-R serum broth.

A similar, if not identical intermediate variant was isolated by chance from subcultures of a single colony of the rough strain D39/R19 by repeated transfer of a loopful of whole culture to fresh 10 per cent anti-R serum broth. At the 14th transfer a faintly turbid supernatant was present and in 53 subsequent transfers almost all of the organisms failed to be agglutinated by the anti-R serum and grew diffusely in the supernatant. This intermediate variant, designated D39/Int53, forms rough colonies on blood agar, is not agglutinated in anti-R serum broth, but is readily agglutinated to high titer by a true anti-SII serum. It appears to be an intermediate between the classical R and S forms of pneumococci. Classical encapsulated S forms, which grow in smooth colonies on blood agar, were not encountered at any time either when supernatants only or whole mixed cultures of D39/R19 were repeatedly transferred in anti-R serum broth. There is no evidence, therefore, that cultures of the rough strain D39/R19 contain any encapsulated Type II pneumococci.

*Production of SSSII by the Intermediate Variant D39/Int53.*—Failure of strain D39/Int53 to be agglutinated when cultivated in anti-R serum broth, even though it still forms a rough surfaced colony on blood agar, suggested that a component is present on the surface of organism of this intermediate variant which is absent from classical R organisms. It seemed likely that the surface component is the Type II polysaccharide, though present in an amount insufficient to give the colonies of D39/Int53 the characteristic smooth, glistening appearance of encapsulated pneumococci. Immunological evidence indicates clearly that the intermediate variant produces SSSII identical with that formed by encapsulated pneumococci of the smooth strain D39/S but in markedly reduced amount.

Antisera prepared in rabbits by immunization with the intermediate variant D39/Int53 gave precipitin reactions with purified Type II capsular polysaccharide diluted to 1:5,000,000 and protected mice against infection with fully virulent Type II pneumococci. Moreover, such sera agglutinated fully encapsulated Type II pneumococci to a titer equal to the best obtained by immunization with the smooth strain itself as shown in Table I. The same sera agglutinated the intermediate variant to a much higher titer. Furthermore, a sample of serum prepared by immunization with the smooth strain D39/S, and repeatedly absorbed with R organisms until it no longer caused their agglutination, maintained its original titer against the smooth strain and also against the intermediate variant. This is also shown in Table I. On the other hand, when antiserum to the smooth strain was absorbed with the intermediate variant, its agglutinating capacity for both strains was removed, as recorded in Table I, and likewise absorption of an anti-intermediate serum

with smooth Type II encapsulated organisms removed the agglutinins for both intermediate and smooth strains. This shows that the specific soluble substance produced by both strains is identical.

The difference in the agglutinating titers of the various sera for the smooth and intermediate strains is of some significance with respect to the amount of SSSII present on the surface of the cells. Titers with the intermediate strain are invariably much higher than with S organisms. The amount of polysaccharide available for combination with antibody is greater in the case of the fully encapsulated smooth strain than with the intermediate variant and, accordingly, the agglutination titer with the smooth strain would be expected to be lower since each cell would be able to combine with more antibody than each cell of the intermediate variant.

TABLE I

*Agglutination Reactions with Antisera to Smooth Type II Pneumococcus Strain D39/S, and to the Intermediate Variant D39/Int53*

Antiserum	Agglutination titers	
	Suspension of smooth strain D39/S	Suspension of intermediate strain D39/Int53
Anti-D39/S (smooth strain)	256	1000
“ absorbed with R pneumococci	256	1000
“ “ “ intermediate variant D39/Int53	0	0
Anti-D39/Int53 (intermediate variant)	256	2000
“ absorbed with smooth strain D39/S	0	0

Absorption of antiserum to the intermediate variant with the fully encapsulated strain D39/S was accomplished relatively easily. However, with the intermediate variant, D39/Int53, repeated absorptions of Type II antiserum with very large amounts of organisms were necessary to remove completely anti-SSSII. Probably this is because of the much smaller amount of SSSII produced by the intermediate variant as compared with the encapsulated strain since the amount of antibody in the two samples of serum was comparable as shown by the agglutination titers recorded in Table I.

Further evidence for the greatly decreased production of SSSII by the intermediate strain is shown by precipitin tests using an antiserum prepared against the smooth strain D39/S from which all anti-R antibodies had been removed by absorption, and culture supernatants of the respective smooth and intermediate variants. The cultures were grown in plain broth for 16 hours at 37°C. There was no obvious difference in the amount of growth of the two strains as indicated by turbidity. The organisms were removed by centrifugation and

dilutions of culture supernatants were added to R-absorbed Type II antiserum diluted in saline in the proportion of 2:3. The results of these precipitin reactions are recorded in Table II.

From the results shown in Table II it can be seen that although the culture supernatant of D39/S when diluted 1:10 gives a well marked precipitin reaction with R-absorbed Type II antiserum, and a trace of precipitation at 1:20, the supernatant of the intermediate variant shows only a trace of reaction when diluted 1:2.

Preparations of purified SSSII in a dilution of 1:2,500,000 gave a definite precipitin reaction with the R-absorbed Type II antiserum used in the tests recorded in Table II.

The question of the quantitative production of SSSII by the intermediate variant was not further explored except for a single attempt to isolate SSSII from the autolysate of a mass culture of D39/Int53 grown in the presence of 1

TABLE II

*Precipitin Reactions of Culture Supernatants of Smooth and Intermediate Organisms with Anti-II S Serum Absorbed with R Organisms*

Strain	Final dilutions of culture supernatant			
	1:2	1:10	1:20	1:200
D39/S (smooth strain)	++++ (disc)	++	Trace	—
D39/Int53 (intermediate variant)	Trace	—	—	—

++ and ++++ indicate the relative intensities of the precipitin reactions.

per cent glucose employing intermittent neutralization. The amount of SSSII, as indicated by precipitin reactions, was so small as to make isolation from other cell constituents impracticable.

*Failure to Demonstrate a Capsular Swelling Reaction with the Intermediate Variant.*—The observations described above indicate that the intermediate variant produces SSSII although in an amount considerably less than that produced by encapsulated organisms. In addition, SSSII would appear to be disposed over the surface of the cells since they are no longer agglutinated by an anti-R serum but are agglutinated by Type II antiserum. It was of interest, therefore, to determine whether capsular swelling could be demonstrated with the intermediate variant.

D39/Int53 was grown under various conditions: in blood broth for 18 hours at 37°C., in broth containing 5 per cent normal horse serum for 4 to 6 hours, and in broth containing 5 per cent glucose for the same period. Pneumococci cultivated under these conditions and obtained from the peritoneal washings of mice that died following injection of 1 cc. of an 18 hour blood broth culture of the intermediate variant were tested for the capsular swelling reaction using

antisera prepared against both the smooth and intermediate variants. In no instance was capsular swelling apparent on repeated test, although it could be seen readily with the same antisera and smooth organisms grown under conditions similar for capsular development. It would appear that although the intermediate variant produced SSSII, and even though the polysaccharide is disposed on the surface of the cells, the amount is not great enough to give the capsular swelling reaction.

*Virulence of the Intermediate Variant for Mice.*—The virulence of the intermediate D39/Int53 was compared to that of the rough strain R36 by injecting mice intraperitoneally with organisms from 18 hour blood broth cultures. The results of this experiment are summarized in Table III. As shown in Table III, 1 cc. of an 18 hour broth culture of D39/Int53 killed all three mice within

TABLE III  
*Virulence of Intermediate Strain, D39/Int53, and an R Strain, R36, for Mice*

Strain	Amount of culture injected	D/S*	Survival time†	Culture of heart blood
	cc.		hrs.	
D39/Int53 (intermediate strain)	1.0	3/0	24	Intermediate variant only
“ “ “	0.5	0/3		
R36 (rough strain)	1.0	2/1	24	R organisms only
“ “ “	0.5	1/2	23	“ “ “

\* Ratio of deaths to survivals.

† Survival time of mice that died.

Surviving mice discarded 7th day.

approximately 24 hours, whereas 0.5 cc. did not cause death. Compared with the encapsulated strain of Type II pneumococcus, D39/S, which kills mice regularly in an amount as small as  $1 \times 10^{-8}$  cc., the intermediate variant is comparatively avirulent. The rough strain, R36, killed 2 of 3 mice when 1 cc. of culture was injected and 1 of 3 mice when 0.5 cc. of culture was injected. It appears, therefore, that the intermediate variant, D39/Int53, is no more virulent for mice than the R strain, R36, despite the fact that it produces SSSII and that the polysaccharide appears to be a surface component. The reason may be that the intermediate variant does not produce the capsular polysaccharide in amounts sufficient to protect itself against phagocytosis.

*Intratype Conversion of R Pneumococci to an Intermediate Variant.*—The foregoing data demonstrate the existence of an intermediate variant of Type II pneumococcus which forms colonies similar in character to those of typical rough organisms, but nonetheless synthesizes the Type II polysaccharide, which appears to be disposed on the surface of the cells although in amount considerably less than produced by the classical S organisms. Since this

intermediate variant remained stable through repeated subcultures in nutrient broth, it seemed of interest to determine whether from the cells a "transforming principle" could be obtained which would be capable of converting typical R pneumococci into variants of the intermediate type.

The technique for preparing transforming extracts has been described under Methods. The R strain used for the transformation reactions, R36NC, is a variant of strain R36 previously used in transformation reactions (7). Strain R36NC does not grow diffusely when cultivated in anti-R serum broth but remains clumped in the bottom of the tubes. The intermediate variant, as noted above, grows diffusely in serum broth. The property of diffuse growth

TABLE IV

*Transformation of R36NC by an Extract of the Intermediate Variant D39/Int53*

Amount of transforming extract of D39/Int53	Turbidity of culture supernatants after 24 hrs. incubation*		Identification of organisms in supernatant
	Original culture	First subculture†	
∞.			
0.2	Trace	++++	Intermediate variant
0.2	"	++++	" "
0.02	"	++++	" "
0.02	"	+	Not studied
None	Clear	Clear	R36NC

\* Trace to ++++ designates degree of turbidity of supernatant, indicating whether transformation has occurred.

† Subculture in 10 per cent serum broth without added transforming extract from D39/Int53.

in anti-R serum broth was used as an indication of whether conversion of R36NC to the intermediate type had occurred in the presence of an extract of intermediate cells, since colonies on blood agar of R36NC and the intermediate variant derived from it appear identical whether viewed with the naked eye or under a colony microscope. In order to prove that conversion to the intermediate type had occurred, reliance had to be placed on serological methods.

Although the means of identifying the converted cells are considerably more difficult than in the case of the transformation of R cells to smooth, encapsulated S forms, it was possible to demonstrate that under the conditions described, R36NC can be converted at will into cells of the intermediate variety. Table IV shows the results of a typical experiment.

No evidence was obtained at any time that strain R36NC was contaminated with intermediate mutants corresponding to D39/Int53 as in the case of the other R strain, D39/R19, from which D39/Int53 was selected. As illustrated in Table IV, however, R36NC could be readily transformed into a strain having the properties of the intermediate variant D39/Int53, originally ob-

tained by selection. Table V shows a comparison of the properties of the original intermediate mutant D39/Int53 and the transformed intermediate mutant R36NC/Int with those of the classical R and S forms of pneumococcus Type II.

In addition to the properties shown in Table V, antisera prepared in rabbits against the respective intermediate variants protected mice to an equal degree

TABLE V

*Properties of Original Intermediate D39/Int53, Transformed Intermediate R36N/Int, and Classical S and R Forms of Pneumococcus Type II*

Properties of pneumococcal strains	Strains of pneumococcus			
	Intermediate D39/Int53 obtained by selection	Intermediate R36NC/Int obtained by transformation	Smooth pneumococcus Type II, strain D39/S	Rough pneumococcus R36NC
Colony morphology on blood agar	Rough	Rough	Smooth	Rough
Growth in anti-R serum broth....	Diffuse	Diffuse	Diffuse	Agglomerated
Reactions in Type II antipneumococcal serum (anti-D39S)				
Agglutination titer.....	1000	1000	256	2000
Quellung reaction.....	Negative	Negative	Positive	Negative
Reactions in antiserum to intermediate variant D39/int53				
Agglutination titer.....	2000	2000	256	8000
Quellung reaction.....	Negative	Negative	Positive	Negative
Reactions in antiserum to intermediate variant R36NC/Int				
Agglutination titer.....	2000	2000	256	8000
Quellung reaction.....	Negative	Negative	Positive	Negative
Character of organisms agglutinated in Type II antipneumococcal serum (anti-D39S).....	Small, hard flakes; difficult to break up	Small, hard flakes; difficult to break up	Hard, disc-like mass	Fine, soft clumps; easily broken up

against infection by the fully virulent encapsulated Type II strain D39S. 0.001 cc. of either antiserum gave protection against approximately 1000 lethal doses of D39S injected intraperitoneally.

Although the properties described for the two intermediate variants indicate strong similarities, it should not be inferred that they are identical in all respects. For example, colonies of the transformed intermediate R36NC/Int resemble closely those of the R strain from which it was derived by transformation, and are different from the colonies of D39/Int53 in certain respects. These colonial differences indicate the presence of systems other than that involved in SSS formation which are not affected in the transformation reaction.



*Conversion of Intermediate D39/Int53 into Fully Encapsulated Type II Pneumococci.*—Attempts to convert the intermediate D39/Int53 to fully encapsulated Type II pneumococci *in vitro* were unsuccessful using either transforming extracts prepared from Type II S or heat-killed cells. It should be recalled that the intermediate variant is not agglutinated when grown in anti-R serum broth, and since growth in agglomerated fashion appears to be one of the prerequisites for transformation *in vitro* (11), it is possible that this accounts for the failure. Other methods for producing agglomeration of the growing organisms, such as cultivation in semisolid agar, were not tried.

Though unsuccessful *in vitro*, conversion of the intermediate D39/Int53 to Type II S was readily accomplished *in vivo* by the original Griffith technique (9) of injecting mice subcutaneously with a mixture containing small numbers

TABLE VI  
*Conversion of the Intermediate Variant D39/Int53 to Fully Encapsulated  
Pneumococcus Type II in Vivo*

Heat-killed pneumococci*	Living pneumococci†	No. of mice injected	No. of mice dying‡	Heart blood culture
Type II S (D39S)	Intermediate D39/Int53	7	5	Pneumococcus Type II
" " "	—	5	0	—
—	Intermediate D39/Int53	7	0	—

\* 0.4 cc. of a thick suspension of Type II pneumococcus heated at 65°C. for 30 minutes.

† 0.1 cc. of an 18 hour blood broth culture.

‡ Average survival time of mice that died was 79 hours; surviving mice were discarded on the 8th day.

of living organisms of the strain to be converted together with large amounts of a heat-killed suspension of smooth organisms. Table VI shows the results of such an experiment. Five of the 7 mice injected with D39/Int53 together with a heat-killed vaccine of Type II S died. From the heart blood of all of these animals fully encapsulated Type II pneumococci were recovered, forming smooth colonies on agar and virulent for mice. Proof of the sterility of the heat-killed suspension of Type II S was obtained not only by injecting mice with 0.4 cc. amounts but also by inoculation into blood broth and on blood agar plates.

*Failure to Transform Type II Intermediate D39/Int53 to Pneumococcus Type III.*—As noted above, the intermediate D39/Int53 could be converted to Type II S *in vivo*, though not *in vitro* by the techniques employed. It was of interest to determine whether it could be transformed to a fully encapsulated heterologous type of pneumococcus. Repeated attempts both *in vitro* and *in vivo* to transform D39/Int53 to pneumococcus Type III were unsuccessful

using extracts of Type III strain A/66 *in vitro*, and heat-killed suspensions of Type III both *in vitro* and *in vivo*. In addition to anti-R serum in the *in vitro* experiments, in certain instances rabbit antiserum to intermediate D39/Int53, previously heated at 65°C. to inactivate desoxyribonuclease, was added in order to cause the intermediate strain to grow in clumps, but without success.

*Conversion of Rough Strain R36NC to Pneumococcus Type II and Transformation to Pneumococcus Type III in a Single Step.*—R36NC is convertible to an intermediate form R36NC/Int, and the intermediate can be converted to fully encapsulated homologous pneumococcus Type II, as shown above. In addition, R36NC can be converted to pneumococcus Type II in a single step by cultivation *in vitro* in the presence of an extract of Type II S. However, it is possible that one or more intermediate phases may appear during the course of the progression from R to S and may be necessary for R—S conversion to occur. Intermediates appearing in the course of "single step" R—S conversion have not been looked for.

The rough strain R36NC can also be transformed to pneumococcus Type III *in vitro*, using transforming extracts prepared from strain A/66.

#### DISCUSSION

The experiments described indicate that in the transformation of an R strain of pneumococcus to the fully encapsulated S form more than a single character may be involved. Mutants intermediate in respect to SSS production occur and conversion can be carried out in two stages, first from R to intermediate and then to the fully encapsulated S form. On the other hand, transformation from R to S may take place in a single step, apparently without the necessity of going through an intermediate phase, although with the usual methods of carrying out transformation reactions, intermediate variants would not ordinarily be detected despite the possibility that they may occur under these circumstances. In this regard it should be recalled that detection of transformation *in vitro* has been based primarily on the gross differences in the appearance of R and S colonies of pneumococcus, so that even though intermediates forming rough colonies were to appear, as in the present studies, they would be missed unless special care were used for their detection.

The SSSII synthesized by the intermediate variant is immunologically indistinguishable from that formed by encapsulated Type II pneumococci as shown by absorption studies. The difference in the strains would seem to reside in the amount of SSSII produced. It is possible that the S gene of the intermediate differs from that of encapsulated Type II organisms in the same respect that alleles for a particular character may differ from each other in higher organisms. On the other hand there may be variation in a separate gene system which exercises control over the amount of SSSII formed by the intermediate as compared with encapsulated pneumococci. An additional

possibility is that the difference between the intermediate and encapsulated cells rests in the permeability of the cell wall to SSSII. An analogy to the latter hypothesis is perhaps to be found in the case of secretors and non-secretors of the blood group-specific substances in man, the capacity to secrete the group-specific substances being transmitted as a Mendelian dominant (12).

It is of considerable interest that although the intermediate variant synthesizes SSSII which is disposed on the surface of the cells, it is not more virulent than R organisms which lack SSSII. It seems likely that the difference in virulence between the intermediate and fully encapsulated pneumococcus Type II is related to the quantity of SSSII synthesized by the respective forms, the intermediate producing an amount insufficient to protect itself against phagocytosis. If this is the explanation of the difference in mouse virulence of these variants, it may be possible in certain instances to account for differences in virulence of various strains of smooth pneumococci of the same serological type on the basis of the quantity of SSS synthesized. From this point of view, enhancement of a potentially virulent strain on repeated mouse passage, for example, would involve selection of variants producing the most SSS. There seems to be little question that virulence is not related solely to the quantity of SSS produced by the various types and strains of pneumococci. The results of the present study indicate, however, that this may be one of the important factors involved.

Virtually complete loss of virulence for mice has not affected to a noticeable extent the capacity of heat-killed suspensions of the intermediate variant to stimulate the production in rabbits of type-specific antibodies reacting *in vitro* with SSSII and protecting mice against infection with the fully virulent smooth organisms. Quantitative estimations of the amount of antibody produced in rabbits have not been made, but the results of precipitin, agglutination, and mouse protection tests indicate that the quantity is comparable to that ordinarily found in the serum of rabbits immunized with similar amounts of fully encapsulated Type II pneumococci. It may be suggested that studies of attenuated strains of bacteria which have been used as immunizing agents for specific prophylaxis might reveal differences in specific cellular components comparable to the reduction in SSSII production of the intermediate variant described. In the case of the intermediate variant the amount of SSSII is insufficient to permit the strain to exhibit significant virulence, but enough is present to act as a potent antigen in rabbits. It should also be possible by techniques analogous to those used in pneumococcal type transformation to "synthesize" bacteria or even viruses having an appropriate combination of avirulence and specific antigenicity.

Failure of the intermediate variant to be transformed to a heterologous type, pneumococcus Type III, may have been due to ignorance of the appropriate conditions. On the other hand, the intermediate already produces SSSII and

if transformation to Type III were to occur, the new strain would be forced to synthesize two capsular polysaccharides at the same time. It seems unlikely that the Type III-synthesizing pattern could be imposed unless that responsible for SSSII production were suppressed or lost through mutation.

In relation to the attempted transformation to pneumococcus Type III, it is of interest that although this transformation did not occur, neither was the Type II intermediate converted into a fully encapsulated strain of pneumococcus Type II by the Type III transforming principle. It would appear, therefore, that the component responsible for *increased* SSSII production in the fully encapsulated SII forms, as compared with the intermediates, is specific for SSSII, and not present in Type III cells.

#### SUMMARY

1. A variant intermediate between the classical R and S forms has been isolated by selective procedures from a rough strain of pneumococcus originally derived from Type II S.

2. The intermediate variant D39/Int53 is avirulent for mice, forms rough colonies, and does not possess a demonstrable capsule. However, it synthesizes SSSII which is immunologically indistinguishable from that produced by fully encapsulated pneumococcus Type II, though in much smaller amount. The polysaccharide is present as a surface component and as it exists in the cell is highly antigenic for rabbits.

3. An extract of the intermediate variant causes the transformation *in vitro* of an R strain into a variant resembling the intermediate in SSSII production but without any apparent alteration in the colonial characteristics of the R variant.

4. The intermediate variant is convertible *in vivo* into a fully encapsulated strain of pneumococcus Type II. Transformation of the intermediate to a heterologous type of pneumococcus (Type III) was unsuccessful.

5. A method is described for the preparation of transforming extracts of pneumococci utilizing the massive growth of the organisms obtained in the presence of a large concentration of glucose.

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# PREPARATION OF HEMOGLOBIN SOLUTIONS FOR INTRA- VENOUS INFUSION\*†§

BY PAUL B. HAMILTON, M.D.,

LEE E. FARR,|| M.D.,

*Lieutenant Commander, Medical Corps, United States Naval Reserve,*

ALMA HILLER, PH.D., AND DONALD D. VAN SLYKE, PH.D.

*(From the United States Navy Research Unit at the Hospital of The Rockefeller  
Institute for Medical Research)*

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The infusion of hemoglobin solutions to replace lost blood has been tested in experiments on both animals (2-15) and man (4, 16-19). Beneficial results have been obtained in restoring the volume of circulating blood, in carrying oxygen to the tissues (3), and in supplying iron for regeneration of erythrocytes and nitrogen for regeneration of blood and tissue proteins (12). For infusions hemoglobin solutions have advantages over whole blood in that the former do not need to be typed and cross-matched, and that longer preservation is possible. However, in both patients and animals, some of the infusions have been followed by unfavorable results, particularly on the kidneys. It has appeared uncertain whether these effects have been due to injury by the hemoglobin itself, caused during its rather rapid excretion through the kidneys, to admixture of methemoglobin with the active hemoglobin (hemoglobin capable of reversible combination with oxygen), to the presence of cell stroma material in the hemoglobin solutions used, or to bacterial contamination or products.

The present work was undertaken to provide a method whereby large volumes of hemoglobin solution could be prepared in a sterile state, free from stroma material, of electrolyte content approximating that of plasma, and capable of preservation without change of the active hemoglobin to methemoglobin.

## *Analytical Methods and Tests*

*The carbon monoxide-binding capacity of the hemoglobin solutions was determined by a modification of the method of Van Slyke and Hiller (20) described by Van Slyke, Hiller,*

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Rockefeller Institute for Medical Research.

† The Bureau of Medicine and Surgery of the United States Navy does not necessarily undertake to endorse views or opinions which are expressed in this paper.

§ Reported in abstract at meetings of the Federated Societies for Experimental Biology and Medicine, Atlantic City, New Jersey, March 14, 1946 (1).

|| On leave of absence from Alfred I. duPont Institute of the Nemours Foundation, Wilmington, Delaware.

Weisiger, and Cruz (21). The hemoglobin fraction capable of binding carbon monoxide is hereafter designated as "active hemoglobin."

*Total hemoglobin content* of the solutions was determined by the above carbon monoxide capacity method after reduction of the solution with sodium hyposulfite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) according to the modification of the method of Van Slyke and Hiller (22) described by Van Slyke, Hiller, Weisiger, and Cruz (21).

The difference between the total hemoglobin content of the solutions and the active hemoglobin content gives the inactive hemoglobin fraction hereafter designated as methemoglobin.

*Total lipid content* of the solutions was determined by extraction of the lipids by the method of Folch and Van Slyke (23) and subsequent estimation of the extracted lipids by determining their carbon by the method of Van Slyke and Folch (24).

The *pH* of the preparations was determined by the glass electrode.

*Sodium and potassium* concentrations of solutions were determined with a flame photometer (25).<sup>1</sup>

Tests for pyrogenic substances, for which we are indebted to Dr. R. B. Pennell of Sharp and Dohme Company, were carried out in accordance with the directions of the U. S. Pharmacopoeia XII (26).

Sterility tests were carried out by culturing 5 ml. aliquots aerobically and anaerobically. We are indebted to Dr. Frank Horsfall, Jr., of the Rockefeller Institute Hospital for most of these tests and to Dr. Pennell of Sharp and Dohme for the remainder.

Toxicity tests were carried out on all earlier preparations. One half ml. of the solution to be tested was injected intraperitoneally into each of ten 15 gm. Swiss mice. The criterion was survival. For later preparations, made after experience gained with the solutions, this test was omitted; but in other experiments the effects of administration to dogs in large quantities intravenously provided evidence for the absence of toxicity factors.

### *Preparation of Hemoglobin Solutions*

The procedure consisted of the following steps: (1) laking the cells with water, (2) precipitation of the stroma material by acidifying to pH 5.7–5.8, (3) ion exchange of most of the potassium for sodium by treatment with sodium zeolite, (4) removal of stroma and zeolite by centrifugation, (5) addition of NaCl and  $\text{NaHCO}_3$  to obtain physiological salt concentration and pH, (6) sterilization by filtration through a Seitz filter.

*General Measures to Insure Sterility.*—Throughout the procedures required in the preparation of the solutions a strict aseptic technique was followed. All containers were sterilized by autoclaving prior to use. When a vessel was opened to receive a solution, the mouth was flamed, and the flaming was repeated before reinsertion of the sterile plug used as a stopper. All water used in preparations was freshly double distilled under conditions making it pyrogen-free. Salts were added to solutions immediately before passage through a Seitz sterilizing filter. All procedures were carried out at 4°C., and the solutions were stored at 4°C., unless otherwise stated. Cultures for aerobic and anaerobic organisms were taken prior to passage through the Seitz sterilizing filter. Bacterial contamination of the solution at this point was

<sup>1</sup> We are greatly indebted to Dr. R. Bowling Barnes and Dr. J. W. Berry of the American Cyanamid Company, Stamford, Connecticut, for the extended use of one of their own research models of the flame photometer, considerably in advance of detailed publication or commercial manufacture of their instruments.



never encountered. The sterility tests were repeated on solutions just prior to use in physiological experiments. Specific procedures are detailed below.

*Source of Red Blood Cells.*—Red blood cell residues discarded from Red Cross donor centers<sup>2</sup> after withdrawal of plasma were used for making human hemoglobin solutions. Solutions of dog hemoglobin were made by bleeding dogs, heparinizing the blood, sterilely aspirating the plasma after centrifugation, and utilizing the residues in the same manner as with human blood.

*Laking of Red Blood Cells and Precipitation of Stroma Material.*—One volume of fresh red blood cell residues was suspended in an equal volume of sterile isotonic saline and repacked by centrifugation for 30 minutes at 3000 R.P.M. The supernatant fluid and buffy layer were aspirated off with aseptic technique and discarded. One volume of washed cell residue was then laked by mixing and standing for 1 hour with two volumes of distilled water. Tenth normal HCl was then added in small portions, usually ten, until the pH of the mixture fell to 5.7–5.8. Approximately 0.6 volume of acid was needed, and it was convenient to add 0.5 volume of acid before determining the pH. During the addition of the acid, the mixture was stirred by swirling the contents of the flask but violent mechanical agitation was avoided; this procedure produced a bulky precipitate which filtered off readily. After addition of the acid, the solution was made up to a volume equal to four times the original volume of red cells used. The 0.1 N HCl used was made up by diluting reagent grade concentrated HCl with pyrogen-free, sterile, distilled water.

*Removal of Excess Potassium.*—Without removing the suspended precipitate, the mixture obtained by the above acidification was treated with sodium zeolite (decalso), to reduce, by ion exchange for Na, the potassium concentration to a physiological plasma level. The decalso, 50 to 80 mesh, was first washed with 5 per cent NaCl solution, dried, and heat-sterilized.<sup>3</sup> Thirty grams of this decalso were added per liter of cell solution, and the mixture was gently stirred several times to insure adequate mixing. Exchange of potassium of the solution with sodium of the decalso approached equilibrium in a few minutes,<sup>4</sup> and, with the amount of decalso used, the concentration of potassium in the treated solution was lowered to approximately 4 milliequivalents per liter.

After the addition of decalso, the mixture was let stand for 1 hour to allow maximum aggregation of the bulky, almost granular precipitate and then the decalso and previously precipitated stroma were removed together by filtration through a coarse filter paper. The filtrate volume approximated 70 per cent of the volume of mixture filtered; the yield by centrifugation was not found sufficiently greater to compensate for using it in place of filtration.

*Adjustment to Final pH and Electrolyte Content.*—The clear deep red filtrate of pH 5.7 to 5.8 contained approximately 4 milliequivalents of potassium and 30 milliequivalents of sodium per liter, chiefly as the chlorides. Solid sodium bicarbonate was added to the solution in an amount sufficiently in excess of the previously added HCl to provide a final concentration of

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<sup>2</sup> The authors are greatly indebted to Dr. Canby Robinson of the American Red Cross for permission to use discarded red blood cell residues, and to Dr. Boynton of the American Red Cross for constant effort to insure that an adequate supply of fresh cell residues was maintained to this laboratory.

<sup>3</sup> Decalso, trade name for a synthetic sodium-aluminum-silicate zeolite, manufactured and obtained from the Permutit Company, 330 West 42nd Street, New York 18, New York.

Drying and heat sterilizing decalso for 1 hour at 160° to 170°C. apparently causes some diminution in its effectiveness in exchanging sodium for potassium; the 30 gm. per liter of solution recommended allows for this slight loss of activity.

<sup>4</sup> That potassium is exchanged for sodium, atom for atom, was established by analysis of the solution for sodium and potassium before and after treatment with decalso.

20 millimolar bicarbonate. In addition, enough solid sodium chloride was added to bring the final concentration of sodium to approximately 145 milliequivalents per liter, and sufficient calcium and magnesium as chlorides to give concentrations of 5 and 2 milliequivalents per liter respectively. The pH of the final solution usually lay between 7.1 and 7.6, depending on the residual amount of carbon dioxide liberated by reaction with the HCl. A sample of the filtrate was taken for sterility tests.

*Sterilization by Seitz Filtration.*—The solution was filtered through a Seitz filter using an S-1 filter pad under 15 to 20 pounds pressure per square inch. Filtration was rapid; 2 liters filter through a pad 120 mm. in diameter in about 15 to 20 minutes. Passage through the Seitz filter also insured removal of any precipitated stroma and zeolite that might not have been removed in the first filtration. The solution was conveniently filtered into 500 ml. or liter bottles, which were then closed with sterile rubber stoppers and kept at 4°C., until used. Portions of the filtrate were taken for sterility and pyrogen tests and additional tests for sterility were done on a number of individual bottles after varying periods of storage.

### *Properties of the Hemoglobin Solutions*

*Composition and Physical Properties.*—Solutions of hemoglobin prepared according to the method just described were clear, of deep red color, and contained approximately 7 gm. of hemoglobin per 100 ml. If the entire procedure was carried out at 4°C., 95 to 98 per cent of the hemoglobin was active. If the procedure was carried out at room temperature with solutions previously chilled to 4°C., and removed from the refrigerator as used, the percentage of active hemoglobin was slightly lower, 92 to 95 per cent. Analysis for lipid carbon showed that 90 to 95 per cent of all lipids had been removed. Numerous tests of human hemoglobin solutions mixed with human blood cells of all four types have never shown any red cell agglutinating properties. No particulate material could be detected on dark field illumination. In the absence of evaporation when kept at 4°C., the solutions remained free of precipitate for 6 months or longer. Some solutions ultimately developed small amounts of a fine reddish precipitate which was observed only after standing 6 months or longer; the formation of precipitate is discussed below.

### *Stability of Solutions Stored at Various Temperatures.*—

Ten ml. portions of a hemoglobin solution were transferred with a sterile pipette into 20 ml. sterile pyrex glass ampules, and the ampules sealed in the oxygen flame. Sealed ampules were stored at 4°C., at 20–25°C. (room temperature), and at 38°C. respectively. From time to time up to 47 days, ampules stored at these temperatures were opened and analyzed for total and active hemoglobin. The results are shown in Fig. 1. It will be seen from these data that the solutions were completely stable at 4°C. but that at room temperature methemoglobin (total—active hemoglobin) formation was appreciable, and that it was still faster at 38°C. It was observed that the formation of precipitate in the solution also depended upon the temperature of storage. Considerable precipitate slowly formed in the solutions stored at 38°C., less in those stored at room temperature, and none was formed in the solutions stored at 4°C. over the 47 day period. Solutions stored under CO<sub>2</sub> at room temperature showed more precipitate than those stored under air at the same temperature.

Two ampules after 6 months' storage at 4°C. showed 3 and 15 per cent formation of methemoglobin respectively. One ampule of hemoglobin solution in which 99.7 per cent of the

active hemoglobin had been deoxygenated by removal of  $O_2$  before the ampule was sealed showed no change in methemoglobin or active hemoglobin after 6 months' storage at  $4^\circ C$ . None of these ampules had developed any sediment in the 6 months' storage period.

Other stability experiments were carried out on 200 cc. lots of hemoglobin solution stored at  $4^\circ C$ . in sterile bottles closed with rubber stoppers. Samples of solution were removed with aseptic precautions at weekly intervals and analyzed for total and active hemoglobin. No methemoglobin formation was observed over a period of 10 weeks; and no sediment was observed at the end of 6 months.<sup>5</sup>

From these experiments it appears that no methemoglobin is formed over a period of  $2\frac{1}{2}$  months; over a 6 month period a small and variable amount of methemoglobin may be formed. Dr. R. B. Pennell of Sharp and Dohme Company<sup>6</sup> has informed the authors that he has succeeded in storing solutions of hemoglobin, prepared in the manner described in this paper, for over 12

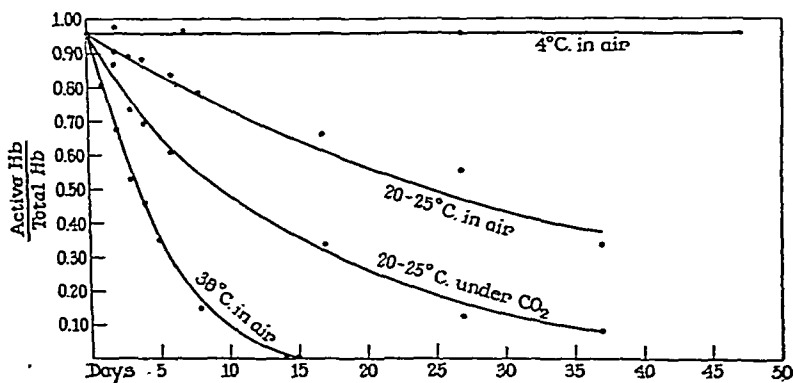


FIG. 1. Decrease in activity of hemoglobin in solutions standing at different temperatures.

months without detectable methemoglobin formation and without any precipitate formation providing the solutions were kept frozen solid at temperatures of  $-5^\circ$  to  $0^\circ C$ . It therefore appears probable that the methemoglobin and sediment formation observed in our experiments was due to storage at too

<sup>5</sup> Anomalous behavior was occasionally noted in a bottle of the thirty or forty that were usually filled from one preparation. Some of these bottles exhibiting anomalous behavior showed on storage fairly rapid conversion of hemoglobin to methemoglobin which on further storage showed slow spontaneous reconversion to active deoxygenated hemoglobin. Other bottles exhibited consumption of oxygen with the formation of deoxygenated hemoglobin without previous conversion to methemoglobin. These changes were always visibly evident, the solution becoming brown with the formation of methemoglobin, and a deep purple hue with the formation of the deoxygenated hemoglobin. Examination of these bottles that behaved anomalously never revealed bacterial contamination.

<sup>6</sup> The authors are indebted to Dr. R. B. Pennell of Sharp and Dohme Company, Glenolden, Pennsylvania, for much helpful information which he has freely placed at our disposal throughout the course of this work.

high a temperature; the minimum temperature of our storage facilities was  $4^{\circ}\text{C}.$ , in refrigerators that were opened frequently and which did in fact suffer considerable elevations of temperature above this minimum as a result of temporary interruption of refrigeration service.

*Effect of Storage under a  $\text{CO}_2$  Atmosphere.*—Ten ml. portions of hemoglobin solutions were transferred, with precautions for sterility, to a few ampules which were then evacuated with a water pump and refilled with  $\text{CO}_2$  four times before sealing of the ampule. These were stored at room temperature only. In these  $\text{CO}_2$ -filled ampules, formation of methemoglobin was more rapid than in the air-filled ampules (Fig. 1). This observation is in keeping with the findings of Brooks (27) who showed that at  $25^{\circ}\text{C}.$  decrease of pH accelerates the conversion of hemoglobin to methemoglobin. On filling ampules with  $\text{CO}_2$  the pH was lowered from 7.6 to 6.8 or 7.0; solutions in ampules sealed with air and stored had pH values of 7.3 to 7.6 when opened subsequently. It is pertinent that Brooks (27) showed that the conversion of hemoglobin to methemoglobin by oxygen under these conditions was the only detectable reaction; *i.e.*, he could not detect conversion of hemoglobin or methemoglobin to other denatured pigments.

#### DISCUSSION

The two major problems which had to be overcome in the preparation of these solutions were the complete removal of stroma and reduction of the potassium content to a concentration approximately that of normal plasma. No adequate tests exist for the detection of stroma material, since the substance has not been sufficiently characterized chemically. We found that simple addition of salt to the laked cell suspension (8, 13, 15, 17, 18) did not remove all material precipitable by toluene or by acid at a pH of 5.9. Cooling the laked cell suspension and bubbling carbon dioxide through it precipitated the stroma material, but the precipitate was so fine that frequently it was impossible to separate it by filtration from the solution, and it blocked a Seitz filter pad. The  $\text{CO}_2$  treatment also increased the proportion of methemoglobin. Removal of stroma with aluminum hydroxide (28) was not found satisfactory. Although toluene as used by Heidelberger (29) and a number of subsequent workers (2-4, 9, 16, 30), appears to remove stroma material quantitatively, the resulting solutions could be rendered free of toluene only with difficulty and uncertainly. Crystallization of hemoglobin and preparation of solutions from such crystallized material was avoided because of the extra steps involved, and because hemoglobin crystallized by Heidelberger's method (29) has been found to contain 7 to 15 per cent of methemoglobin (31). The acidification technique originally described by Jorpes (32) for the isolation of stroma material was found to be efficient and easily adaptable to the present investigation. However, it was found that if the HCl is added slowly as from a burette with vig-

orous mechanical stirring a very fine precipitate forms, presumably stroma, which is very difficult to centrifuge down and impossible to filter off, even though the pH has been brought to 5.7 to 5.8. If to this unfilterable suspension of stroma material 1 to 5 per cent of acid-denatured hemoglobin is added rapid and complete flocculation of the stroma occurs in a form that can be readily filtered off. Since the technique of acidification used in our preparations produced an easily filterable precipitate it is inferred that there must have been small amounts of acid-denatured hemoglobin formed on the addition of the HCl. These observations indicate that adjustment to the isoelectric point of stroma is not the only factor involved in the precipitation of stroma on the addition of acid.

Although removal of excess potassium in solutions made from dog hemoglobin is not necessary because of the low potassium content of red blood cells of the dog, it is required with solutions made from human red blood cells, or the resulting solution will have a potassium content close to toxic levels (33) for plasma. Ion exchange with sodium zeolite proved to be a rapid and economical means of reducing the  $K^+$  concentration to normal physiological levels by replacement with  $Na^+$ .

The importance of keeping bacterial contamination and growth low hardly needs emphasis, for toxic substances are rapidly produced when bacteria multiply in solutions of hemoglobin. Most investigators previously have preferred to prepare solutions for immediate use, sacrificing rigid aseptic technique for speed of preparation and relying on low temperatures to retard bacterial growth till the moment of injection. None have mentioned carrying out tests for pyrogenic substances, and in the absence of such tests it is difficult to assay reactions, such as chills and fever, which have been reported to follow intravenous administration of hemoglobin solutions into human subjects (13, 15-18).

Deoxygenated homoglobin provides a more stable form than oxyhemoglobin for storage in solution. However, complete deoxygenation is a long and tedious process, and since the oxyhemoglobin solutions have been found to be stable at 4°C. over a period of at least 6 months, we do not believe that the advantages to be gained by deoxygenation are equivalent to the increased labor of preparation. Hemoglobin solutions prepared for freezing and drying must be completely deoxygenated, as noted in the accompanying paper (34) to prevent methemoglobin formation during the process of dehydrating the frozen mixture.

#### SUMMARY

A procedure has been detailed for the preparation of sterile non-pyrogenic solutions of oxyhemoglobin which have the approximate protein content and electrolyte composition of plasma.

Large volumes of solution can be rapidly prepared, with 95 to 98 per cent of the hemoglobin in the active form capable of combining with oxygen. The

solutions contain no particulate matter; 95 per cent of total blood lipids are removed.

Solutions stored at 4°C. showed no conversion of hemoglobin to methemoglobin over a period of 2½ months; over a 6 month period a small and variable amount of methemoglobin may be formed.

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# PREPARATION OF DRIED HEMOGLOBIN WITHOUT LOSS OF ACTIVITY\*†§

BY LEE E. FARR,|| M.D.,

*Lieutenant Commander, Medical Corps, United States Naval Reserve,*

ALMA HILLER, PH.D., AND DONALD D. VAN SLYKE, PH.D.

WITH THE TECHNICAL ASSISTANCE OF JOHN PLAZIN

*(From the United States Navy Research Unit at the Hospital of The Rockefeller Institute for Medical Research)*

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As an extension of the study of the properties and characteristics of hemoglobin solutions prepared as detailed in an accompanying paper (2), further experiments were carried out to determine whether such solutions could be frozen and dried without change in hemoglobin activity, and to determine the stability of such dried preparations under selected conditions as compared with the stability of the original solutions.

Morrison and Hisey (3) have shown that if a hemoglobin solution is completely deoxygenated it can be dried without formation of methemoglobin, and Drabkin (4) has shown that hemoglobin can be dried by the "lyophile" procedure without denaturing the protein. In the present work we have utilized this principle, applying the technique of first freezing and then drying *in vacuo* as utilized with plasma in order to obtain the dried material in a honeycomb structure capable of being quickly redissolved. The procedure has not been applied to large scale production, but the conditions have been defined that are necessary for preparation by its means of dried hemoglobin of minimal methemoglobin content.

## *Material and Methods*

The vessel in which the hemoglobin solution was deoxygenated was of the type shown in the upper part of Fig. 1. It had at one end a two-way cock, at the other end a three-way cock with an 8 cm. length of heavy wall capillary tubing drawn at the tip to permit its insertion into a rubber tip which fits into the cup of the Van Slyke-Neill manometric gas apparatus (5). A

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|| On leave of absence from Alfred I. duPont Institute of The Nemours Foundation, Wilmington, Delaware.

three-way stopcock permits connection of the capillary delivery tube with either the contents of the vessel or a side arm connecting tube. For analysis of hemoglobin solution in the vessel, the cleaned and dried tip is placed in the cup of the Van Slyke-Neill gas apparatus under mercury, and mercury from the chamber is run up through the capillary delivery tube and into the side arm tube, thereby effectively displacing all air from the delivery tube system. The stopcock is then turned to admit the sample to a calibration mark previously made on the chamber so that the volume analyzed is 2 ml.

*Deoxygenation of Oxyhemoglobin Solutions.*—By repeated shaking under diminished pressure, oxygenated hemoglobin solutions were deoxygenated. Removal of all but the slightest traces of oxygen was accomplished by alternately de-gassing the solutions and saturating them with oxygen-free nitrogen. The latter gas was prepared oxygen-free by shaking with and storage over an alkaline pyrogallol solution.<sup>1</sup> Usually 40 ml. of the solution to be deoxygenated were transferred to an 800 ml. vessel of the type shown in the upper part of Fig. 1. By means of a Hy-Vac pump, pressure within the vessel was reduced gradually while the vessel was vigorously shaken. When frothing became severe the pressure was kept constant until with very vigorous shaking the solution became foam-free after which further pressure reduction was effected. The final pressure was 10 mm. of mercury. The solution in the vessel was adjudged to be gas-free when a distinct metallic ring was emitted upon shaking. At this time the vessel was refilled to slightly above atmospheric pressure with oxygen-free nitrogen and the procedure repeated. After four to six repetitions of evacuation to a gas-free state, the vessel was refilled with nitrogen and a sample of solution was removed for analysis of oxygen content. During the process of deoxygenation, approximately 20 per cent of the water of the solution was evaporated, and the final hemoglobin concentration was 7 to 9 per cent or greater. If, upon analysis, the oxygen content was 0.3 volume per cent or less the solution was considered to be sufficiently deoxygenated for freezing; an analysis was made for total and active hemoglobin and the preparation was ready for freezing. The entire process of deoxygenation required an average of 4 hours of continued careful and watchful manipulation.

Attempts to replace mechanical removal of oxygen by enzymic reduction were not successful.

*Freezing and Drying of Deoxygenated Hemoglobin*—The deoxygenated hemoglobin solutions were transferred without contact with air to a mercury-filled vessel connected to a reservoir of mercury. The vessel was then connected by means of heavy walled rubber tubing to a 25 ml. ampule. The ampule and connecting system were evacuated and filled with oxygen-free nitrogen three times; then 5 ml. of hemoglobin solution were transferred from the vessel to the ampule. The heavy walled tubing was securely clamped and the ampule and tube removed from the system. The ampule was then immersed in a bath of dry ice and alcohol and rapidly rotated until the hemoglobin solution had frozen. After this the ampules were connected to the drying apparatus, the clamp removed from the rubber tubing, and the frozen preparation was dried for 4 to 5 hours at less than 20 microns pressure. Upon completion of drying, the evacuated ampules were sealed with an oxygen flame and the preparation stored at 4°C. until required for other testing.

*Redissolving of Deoxygenated Dried Hemoglobin.*—It was found that even momentary contact of the dried reduced hemoglobin with atmospheric oxygen resulted in measurable methemoglobin formation. It was therefore necessary to exclude air completely during resolution of the hemoglobin. Once it had been dissolved, as was found by Morrison and Hisey (3)

<sup>1</sup> The nitrogen commercially available in tanks contains sufficient oxygen to prevent obtaining the degree of deoxygenation required. It was therefore made oxygen-free in a reservoir of pyrogallol from which it was then used.

access of air did not cause measurable methemoglobin formation. The apparatus shown in Fig. 1 was used for dissolving the dried deoxygenated hemoglobin without contact of the dry preparation with air.

When oxygen-free water was used to dissolve the dried hemoglobin, 10 ml. of distilled water were made air-free in the chamber of the Van Slyke-Neill gas apparatus, then trans-

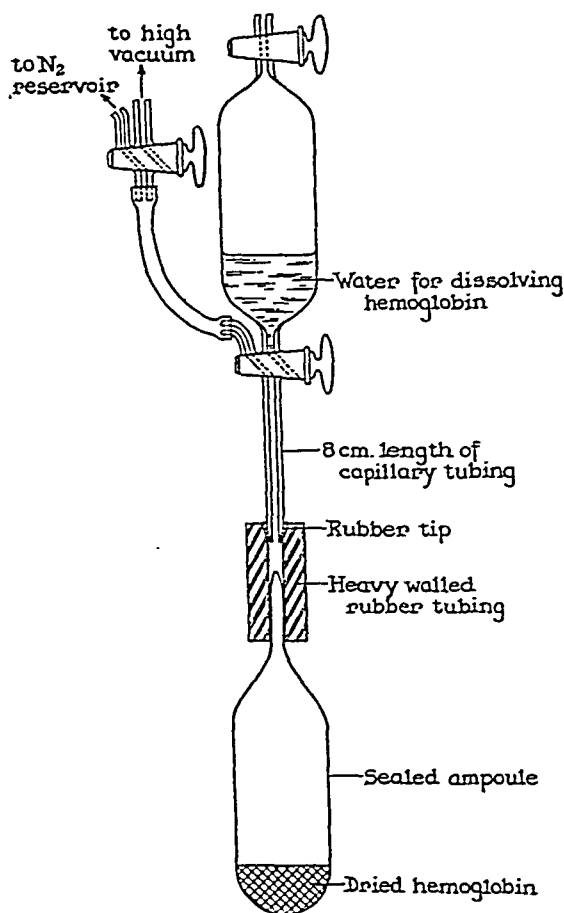


FIG. 1. Apparatus for dissolving dried hemoglobin without contact with air.

ferred to the upper vessel of Fig. 1 which had been previously evacuated to high vacuum and filled with oxygen-free nitrogen. The vessel was then connected with the sealed ampoule by means of heavy walled rubber tubing as shown in Fig. 1. The dead space between ampoule and vessel was alternately evacuated and filled with oxygen-free nitrogen to wash out the air. The cock of the vessel containing water was then turned to connect the vessel with the ampoule, and the ampule tip was crushed by pressing the rubber tubing with heavy pliers. The water entered the evacuated ampoule with considerable force, and solution was immediate. Without disconnecting, the apparatus was inverted and the solution of hemoglobin transferred from

ampule to vessel by alternate evacuation of the vessel and admission of nitrogen into the ampule. When all of the hemoglobin solution was in the vessel in an atmosphere of nitrogen, the vessel was disconnected. The tip was washed and dried through the side arm, and samples of the hemoglobin solution were measured directly into Van Slyke-Neill chambers for determination of oxygen content. The remainder of the solution was analyzed by the usual technique (6) for active and total hemoglobin, samples being measured from pipettes as usual (6).

In the experiments in which the dried hemoglobin was dissolved in air-saturated water, 10 ml. of the water were placed in the vessel (upper part of Fig. 1) without evacuating the vessel, and admitted to the ampule as described above, so that the dried hemoglobin *was covered with water before air entered the ampule*. Under these conditions, as shown by the last two columns of Fig. 4, resolution of the hemoglobin occurred without methemoglobin formation.

*Freezing, Drying, and Redissolving of Oxygenated Hemoglobin.*—Solutions of oxygenated hemoglobin were frozen in the usual manner in a bath of dry ice and alcohol, then dried, redissolved in water, and analyzed for total and active hemoglobin. The effects of varying quantities of added electrolytes were studied. In these experiments, the dried preparation was not stored, but was immediately redissolved and analyzed.

### *Analysis of Hemoglobin Solutions and Dried Preparations*

*Determination of Small Amounts of Oxygen in Solutions of Deoxygenated Hemoglobin.*—In determining oxygen in almost completely deoxygenated hemoglobin solutions it was found necessary, needless to say, to avoid the slightest contact of air with the hemoglobin solution. For this reason samples could not be transferred to the manometric apparatus by the usual method of pipetting. The gasometric method of Van Slyke and Neill (5) for the determination of oxygen was therefore modified to permit transfer of measured samples of deoxygenated hemoglobin solutions directly from the vessel in which the deoxygenation was performed into the chamber of the manometric apparatus. The usual order of first measuring dilute ferricyanide solution into the chamber, extracting the air from it, and then measuring the blood sample, was of necessity reversed. The hemoglobin solution was first measured into the chamber, the sample being measured by a 2 ml. mark on the chamber; then 3 ml. of completely deaerated water from a mercury-sealed vessel (Fig. 2) was measured in, followed by 0.3 ml. of 32 per cent ferricyanide. In using this reversed procedure the chamber must be made completely air-free before admitting the deoxygenated sample.

Calibration of the chamber to receive a 2 ml. sample was effected by admitting into the chamber 2 ml. of water from a pipette provided with a rubber-ringed tip which was pressed through about 0.5 ml. of mercury in the cup of the chamber. The stopcock of the chamber was closed without admitting mercury from the cup so that the 2 ml. of water occupied the capillary below the cup and the chamber to a point a little above the usual 2 ml. mark. The level of the mercury meniscus was marked on the chamber.

Before analysis the chamber of the manometric apparatus was freed of air by extracting the dissolved gases from a few milliliters of water in the evacuated chamber and ejecting the evolved air and the water. Approximately 1 ml. of mercury from the chamber was then run up into the cup. The rubber-ringed tip of the vessel (upper part of Fig. 1) containing the deoxygenated hemoglobin solution was pressed into the bottom of the cup of the chamber under mercury. Mercury from the chamber was run up through the capillary delivery tube of the vessel (Fig. 1) and into its side arm, displacing air from the delivery tube. Then 2 ml. of the hemoglobin solution were admitted into the chamber to the temporary calibration mark made on the chamber, followed by 3 ml. of air-free water from the vessel shown in Fig. 2. The mercury was removed from the cup and two drops of caprylic alcohol were admitted into the capillary below the cup. Approximately 0.5 ml. of water was then placed in the cup and 0.3 ml. of the 32 per cent potassium ferricyanide was delivered into the chamber from a rubber-

tipped burette graduated in 0.01 ml., the tip of the burette being pressed under the water into the bottom of the cup above the Van Slyke-Neill chamber. The rest of the oxygen determination was carried through as described by Van Slyke and Neill (5).

*Determination of Active Hemoglobin and Total Hemoglobin.*—Active hemoglobin (capable of binding CO) and total hemoglobin were determined in the solution by the carbon monoxide capacity method as described by Van Slyke, Hiller, Weisiger, and Cruz (6).

*Calculations.*—The difference between the total hemoglobin content of the solutions and the active hemoglobin content is calculated as the inactive hemoglobin fraction hereafter designated as methemoglobin.

Per cent active hemoglobin was calculated as  $100 \times \frac{\text{Active hemoglobin}}{\text{Total hemoglobin}}$ .

Per cent deoxygenated hemoglobin was calculated as  $100 - \frac{\text{O}_2 \text{ content} \times 100}{\text{CO capacity}}$ .

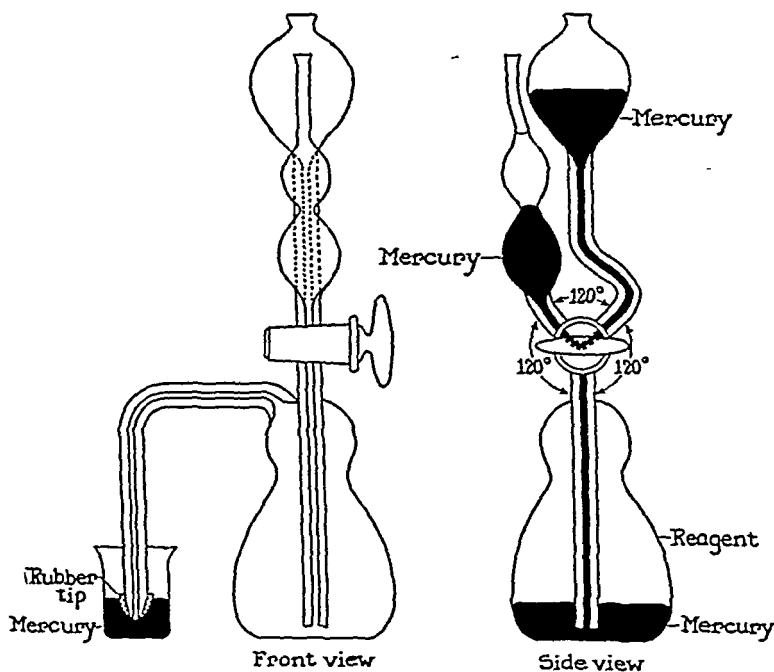


FIG. 2. Apparatus for storing air-free reagents over mercury and for delivering measured amounts into chamber of the Van Slyke-Neill gas apparatus.

### *Results with Oxygenated Hemoglobin Solutions*

*Effect of Freezing and Thawing.*—Solutions of oxygenated hemoglobin which had been analyzed for active and total hemoglobin were frozen quickly by immersion in a bath of dry ice and alcohol, then permitted to thaw at room temperature and again analyzed for active hemoglobin. The results of such an experi-

ment are shown in Fig. 3. Freezing and thawing caused no change in the activity of oxyhemoglobin solutions.

*Effect of Freezing and Drying Oxygenated Hemoglobin Solutions.*—Solutions of oxygenated hemoglobin which had been analyzed for active and total hemoglobin were frozen, dried, redissolved in water, and again analyzed for total and active hemoglobin. The results obtained in a typical experiment of this kind are shown in Fig. 3. In all experiments of this nature, 20 to 30 per cent of the original active hemoglobin was changed to methemoglobin. Precautions taken to exclude air at time of redissolving did not change the activity. Exposure of the dried oxyhemoglobin to air for 3 minutes at room temperature and for 24 hours at 4°C. before dissolving did not alter the activity. The

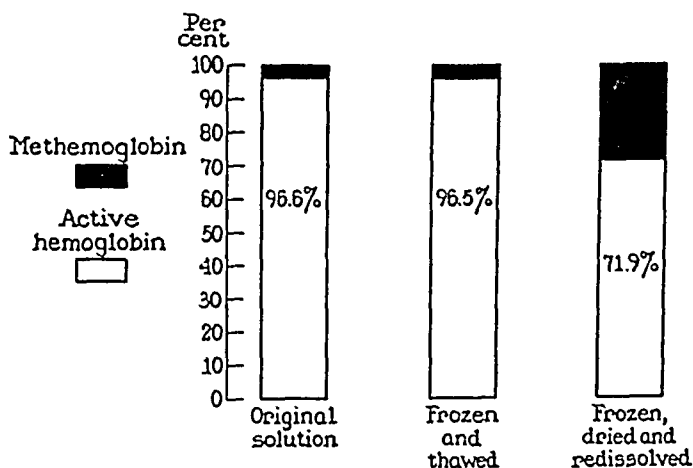


FIG. 3. Effect of freezing and drying on oxygen-binding capacity of oxyhemoglobin

addition of electrolytes to concentrations usually found in plasma slightly increased the yield of active hemoglobin. The addition of sodium oleate did not affect the formation of methemoglobin.

#### *Results with Deoxygenated Hemoglobin Solutions*

*Effect of Deoxygenation on the Activity of Hemoglobin Solutions.*—Deoxygenation of solutions of oxyhemoglobin caused no change in the activity measured by CO-binding capacity. The results of a typical experiment are shown in Fig. 4. In this experiment 99.7 per cent of the oxyhemoglobin was deoxygenated. The hemoglobin went through the procedure of repeated vacuum extraction of the gases without losing as much as 1 per cent of its CO-binding activity. In no such experiment was there a loss of activity greater than 1 per cent.

*Effect of Freezing, Drying, and Redissolving of Deoxygenated Hemoglobin Solutions.*—When dried preparations of deoxygenated hemoglobin kept in

*vacuo* were dissolved in oxygen-free water in an atmosphere of nitrogen the activity of the resulting hemoglobin solution was the same as that of the deoxygenated solution before freezing and drying, as shown in Fig. 4. Analyses of oxygen content showed that the resulting solutions were between 99 and 100 per cent deoxygenated.

When dried preparations of deoxygenated hemoglobin kept *in vacuo* were dissolved in water containing air, as described previously, in such a way that the air was removed from the space between the ampule and the vessel containing water before the ampule was opened, solution of the dried powder was immediate and resulted in a preparation of the same activity as that of the original solution of deoxygenated hemoglobin as shown in Figs. 4 and 5. The hemo-

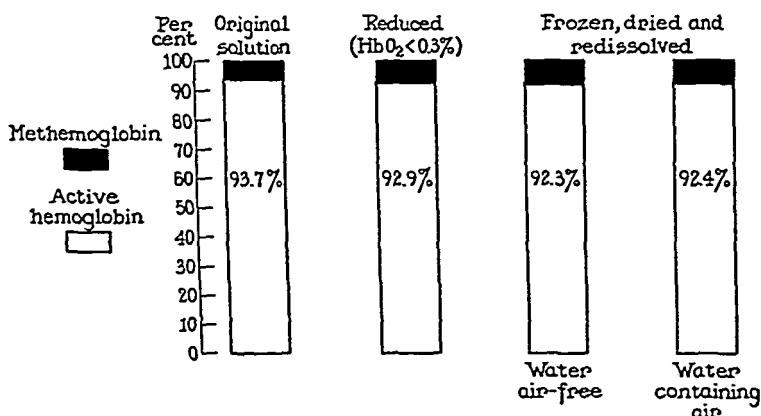


FIG. 4. Effect of freezing and drying on oxygen-binding capacity of reduced hemoglobin.

globin, after solution had been effected, could be exposed to either air or a stream of pure oxygen without any increase in the methemoglobin content of the solution. The results of three such experiments are shown in Table I.

When, however, the dried deoxygenated hemoglobin was exposed to air for 3 minutes before dissolving in water, the activity of the resulting solution was reduced by 33 per cent as shown in Fig. 5. This reduction in activity occurred whether the water was oxygen-free or saturated with air. When the dry product was exposed to air at 4°C. for 1 month before dissolving, the activity fell 60 per cent. Even momentary exposure of the dried deoxygenated hemoglobin to air at reduced pressure with subsequent rapid evacuation of admitted air, was followed by a very significant reduction in the active hemoglobin fraction amounting to approximately 10 per cent of the active hemoglobin originally present.

Once the dried deoxygenated hemoglobin had been dissolved, an increase in methemoglobin did not occur with exposure to air or oxygen (Table I) and the

concentration of active hemoglobin remained constant over a period of weeks if kept at temperatures of 4–6°C. All solutions dissolved by any of the three procedures used above were reanalyzed after a period of 3 weeks at 4–6°C.

TABLE I

*Effect of Oxygenation of Redissolved Reduced, Frozen, and Dried Hemoglobin Solutions on Activity*

State of solution	Oxygen content	Per cent deoxygenated	Total hemoglobin	Active hemoglobin	Activity
	vol. per cent	per cent	gm. per 100 ml.	gm. per 100 ml.	per cent
Before oxygenation.....	0.06	99.1	5.41	5.07	93.6
After oxygenation.....					
Before oxygenation.....	0.02	99.8	6.13	5.81	94.8
After oxygenation.....					
Before oxygenation.....	0.03	99.7	7.11	6.76	95.0
After oxygenation.....					

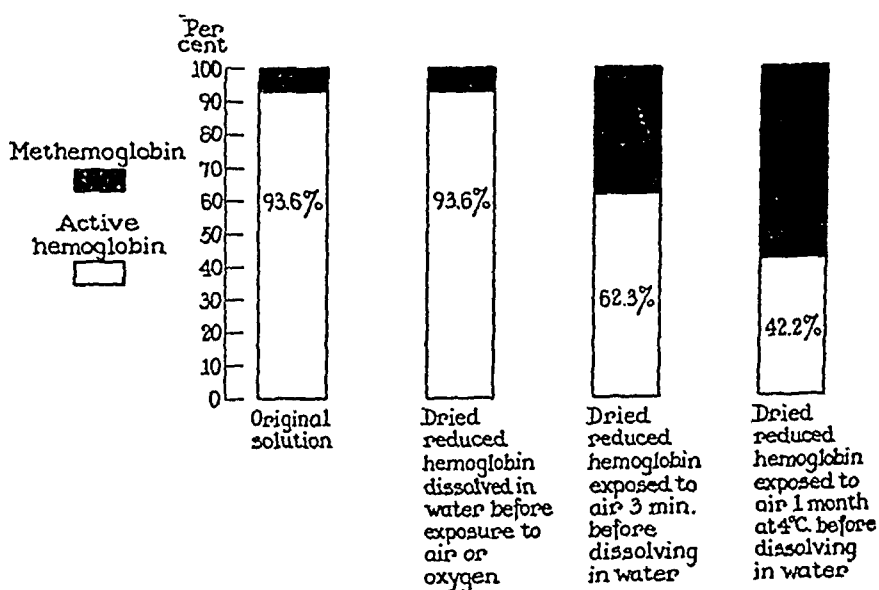


FIG. 5. Effect of air (oxygen) on oxygen-binding capacity of dry reduced hemoglobin.

and were always found to have the same quantity of active hemoglobin as when they were initially dissolved.

*Stability of Dried Deoxygenated Hemoglobin Stored in Vacuo at Various Temperatures.*—Ampules of dried deoxygenated hemoglobin sealed *in vacuo* were stored at 4°C. for periods of time up to 180 days, then dissolved in oxygen-free water and analyzed. In each instance there was no decrease in active hemo-



globin in any of the samples so tested over the entire 180 day period. One such experiment is shown in Fig. 6. Ampules stored at room temperature, which varied between 20° and 30°C., were removed at intervals and the dried hemoglobin was redissolved in oxygen-free water and analyzed. Again no deterioration of the product was noted with time. One such experiment in which the ampule had been stored for 180 days is shown in Fig. 6. The hemoglobin solution was found to be 99.7 per cent deoxygenated and had an activity identical with that of the original solution from which the dried product was prepared. An ampule stored at 38°C. for 1 month before dissolving was found to be 99 per cent deoxygenated, with no loss in activity. Another ampule stored at 38°C. for 92 days was found to be 87.9 per cent deoxygenated, with

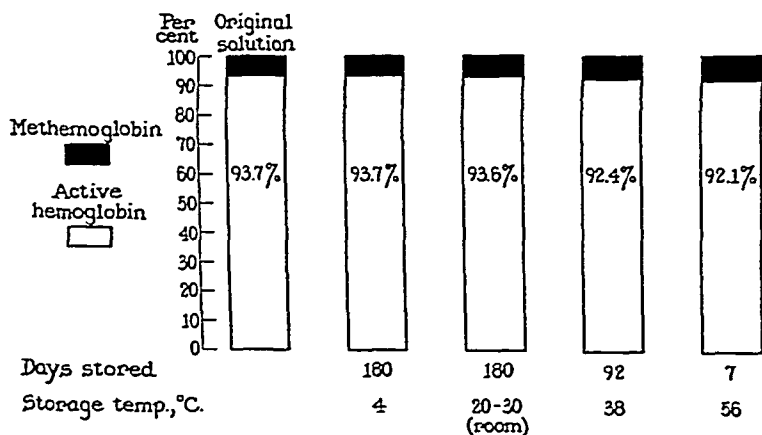


FIG. 6. Stability of dried reduced hemoglobin stored *in vacuo*.

an activity 98.8 per cent of the original solution. This is shown in Fig. 6. We believe the apparent decrease in the percentage of deoxygenated hemoglobin in this one instance was an artifact and occurred during the process of redissolving. One ampule stored at 56°C. for 7 days was found to be 99 per cent deoxygenated and had an activity of 98.3 per cent of the original solution (Fig. 6).

#### DISCUSSION

That crystalline oxyhemoglobin cannot be dried without loss of activity was first shown by Bohr (7) in 1892. Subsequently attempts to dry crystalline oxyhemoglobin reported by Hufner (8), Heidelberger (9), Van Slyke, Hastings, Heidelberger, and Neill (10), and Barcroft (11) were uniformly unsuccessful, as either a very large fraction or all of the hemoglobin was converted into methemoglobin by the procedure. Cannan and Redish (12) were unable to prevent from 40 to 60 per cent of the hemoglobin from being converted into methemo-

globin by drying either (1) "*in vacuo* from the frozen state," or (2) "by addition of anhydrous sodium sulfate to the crystal cake" or (3) "by air-drying a concentrated solution in a cellophane bag." Amberson, Jacobs, Hisey, and Monke (13) found on freezing and drying oxyhemoglobin solutions that methemoglobin constituted half or more of the total pigment. When the solutions were degassed prior to freezing, methemoglobin formation was definitely decreased, although far from being suppressed. When glucose was added to 5 per cent concentration to the hemoglobin solutions before freezing and drying, the amount of methemoglobin formation was reduced to about 20 per cent of the total pigment. Such solution, however, could not be completely dried. To secure a dry product only 1 or 2 per cent glucose was added and under these conditions up to 25 per cent of the hemoglobin changed to methemoglobin. Our experience with oxyhemoglobin has been similar.

In 1937 Morrison and Hisey (3) found that if hemoglobin solution was dried by distilling off the water *in vacuo* at 38°C., and was redissolved without contact with air the average loss of oxygen-binding activity was only 3 per cent. Morrison and Hisey demonstrated that oxyhemoglobin in the dry state forms methemoglobin much more rapidly than does oxyhemoglobin in solution, and that removal of oxygen, which they accomplished by distillation, is a prerequisite to drying without methemoglobin formation.

After this work was finished, Pennell, Smith, and Werkheiser (14) published a procedure for removing the oxygen from oxyhemoglobin solutions which appears better adapted to large scale preparations. These authors obtain complete deoxygenation by action of the enzymes in laked cells after addition of nicotinic acid and glucose.

#### SUMMARY

The technique for freezing, drying, and preserving *in vacuo* which is in common use for plasma can be successfully applied to hemoglobin solutions when the hemoglobin is first deoxygenated to the extent of 99.7 per cent or more.

In confirmation of Morrison and Hisey, the preliminary deoxygenation of the solution is found necessary to avoid formation of methemoglobin during drying. If a solution of oxyhemoglobin is frozen and dried, 20 to 30 per cent is changed to methemoglobin.

Deoxygenated hemoglobin dried and preserved *in vacuo* retained all its oxygen-binding activity for 180 days, when stored at temperatures from 4° to 30°C. Storage at 38°C. for 92 days, or at 56° for 7 days, caused no loss in activity. The dried hemoglobin had a foam structure which caused it to dissolve immediately upon contact with water.

Deoxygenated hemoglobin in the dry state was partly converted to methemoglobin by even momentary contact with oxygen. When, however, the deoxygenated hemoglobin was dissolved *before* it was exposed to air, the hemoglo-

bin in solution was relatively stable, and could be stored for months at 4° in contact with air without significant loss of activity.

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## RENAL EFFECTS OF HEMOGLOBIN INFUSIONS IN DOGS IN HEMORRHAGIC SHOCK\*†

BY PAUL B. HAMILTON, M.D., ALMA HILLER, PH.D., AND  
DONALD D. VAN SLYKE, PH.D.

*(From the United States Navy Research Unit at the Hospital of The Rockefeller  
Institute for Medical Research)*

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The most serious deterrent to the intravenous use of hemoglobin solutions has been the fear of renal damage such as has been observed to occur following its use, as also after transfusion reactions. Chills and fever have also been noted in man, but these reactions have proved of minor significance. The untoward results have been variously attributed to red cell stroma, abnormal concentrations of potassium, bacterial contamination, chemical changes in the hemoglobin, and even referred to the hemoglobin itself.<sup>1</sup>

Attention was first directed by Baker and Dodds (3) to changes in kidney function resulting from the intravenous injection of hemoglobin solutions into rabbits. These authors concluded that hemoglobin, which readily passes through the glomerular membrane, is precipitated in the kidney tubules where concentration of the glomerular filtrate takes place. They believe that two factors, increase of acidity and increase of salt concentration, together caused precipitation of the hemoglobin, and that in turn this precipitated hemoglobin mechanically plugged the tubule lumens. As a consequence of this plugging, renal function was damaged; a rise in blood urea nitrogen was taken as evidence of this damage. De Nevasquez (4) disagreed with the conclusions of Baker and Dodds when he found that for rabbits, in spite of elevated blood urea nitrogen, renal function as judged by the excretion of phenol red, remained normal regardless of the acidity of the urine following the intravenous injection of hemoglobin. De Gowin, Osterhagen, and Andersch (5) injected laked dog red blood cells (stroma not removed) into dogs and appeared to confirm the findings of Baker and Dodds, but on examination of stained sections of kidneys from these animals, De Gowin, Warner, and Randall (6) were led to state that although plugging of the renal tubules was observed there was an unexplained nephrotoxic process which could cause renal

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† The Bureau of Medicine and Surgery of the United States Navy does not necessarily undertake to endorse views or opinions expressed in this paper.

<sup>1</sup> For bibliography pertaining to the various methods of preparing solutions of hemoglobin see Hamilton, Farr, Hiller, and Van Slyke (1). For bibliography pertaining to the intravenous administration of hemoglobin solutions to animals and man see Farr, Hiller, and Van Slyke (2).

insufficiency, independent of and in the absence of mechanical obstruction of the tubules by precipitated hemoglobin. Bing (7) investigated renal function of normal and acidotic dogs receiving hemoglobin injections and concluded that hemoglobin and methemoglobin in the normal dog and hemoglobin in the acidotic dog gave no depression of renal function as measured by clearance of para-amino hippuric acid and creatinine. He did find, however, that methemoglobin caused marked depression of kidney function when injected into acidotic animals. It appears that in Bing's experiments with methemoglobin and acidosis the animals suffered other damage in addition to depression of kidney function, because the dogs were usually moribund by the 3rd day after receiving the injection. As a rule, dogs do not become moribund from renal failure alone till the 4th to 8th day, even after surgical removal of both kidneys (8, 9).

In an attempt to gain further insight into the renal effects of treating shock by intravenous injection of hemoglobin solution the writers have infused solutions of dog hemoglobin prepared by the method of Hamilton, Farr, Hiller, and Van Slyke (1) into dogs in hemorrhagic shock. Renal function was investigated before and after replacing 50 cc. of blood per kilo body weight by an equal volume of approximately 7 per cent hemoglobin solution.

#### *Preparation of Plasma and Hemoglobin Solutions for Infusion*

Blood was collected from normal dogs in sterile 100 cc. centrifuge bottles containing 50 mg. heparin and centrifuged. The plasma was siphoned off, pooled, sterilized by passing through a Seitz filter, and stored in sterile bottles at 4°C.

From the residual red blood cells, solutions of hemoglobin were prepared according to the procedure described in an accompanying paper (1). The hemoglobin of the solutions was 95 to 98 per cent in the active form capable of carrying oxygen. Methemoglobin solutions were prepared by adding 1.1 moles of sodium ferricyanide per mole of hemoglobin. Analysis showed that at least 99 per cent of the hemoglobin was in the inactive form, incapable of carrying oxygen; *i.e.*, as methemoglobin.

#### *Methods of Analysis*

*Total hemoglobin* content of the injected hemoglobin solutions and of plasma and urine of dogs after hemoglobin injection was determined by the carbon monoxide capacity method, with addition of  $\text{Na}_2\text{S}_2\text{O}_4$  to reduce any ferrihemoglobin to ferroheminoglobin ("active Hb"), according to the method of Van Slyke and Hiller (10) as modified by Van Slyke, Hiller, Weisiger, and Cruz (11).

*Methemoglobin* content of injected solutions and of plasma and urine of dogs after injection was calculated as the difference between total hemoglobin and active hemoglobin (11).

*Blood plasma urea nitrogen* was determined by the hypobromite method of Van Slyke and Kugel (12).

*Urine urea nitrogen* in the control periods, before hemorrhage, and in the earlier experiments, Figs. 1, 2, and 5, was determined by the hypobromite method of Van Slyke (13) but using the hypobromite reagent described by Van Slyke and Kugel (12). In the later experiments, Fig. 7, urine urea nitrogen was determined by the urease-aeration method of Van Slyke and Cullen (14) and in Figs. 3 and 6 by the gasometric urease method of Van Slyke (15).

*Plasma chlorides* were determined by the method of Van Slyke and Hiller (16).

*Plasma  $\text{CO}_2$*  was determined by the method of Van Slyke and Neill (17, 18). The corrected factors of Van Slyke and Sendroy (19) were used to calculate the values for plasma  $\text{CO}_2$ .

### *Experimental Procedures*

In the initial experiments animals were used after an 18 hour period of fasting. In later experiments all animals were placed for periods of 7 to 10 days in metabolism cages. Daily morning fasting venous blood samples were drawn for determination of plasma urea nitrogen. Twenty-four hour urine excretions were also collected in large flasks containing thymol and completely immersed in crushed ice in order to keep bacterial growth minimal or absent. The 24 hour output of urine urea was determined, and from the values of blood and urine urea the 24 hour urea clearance values  $\left(\frac{UV}{B}\right)$  (20) were calculated. This procedure was adopted because it eliminated the necessity of training animals to submit to catheterization. Female dogs were used in all experiments and were kept on stock laboratory diets.

Animals were last fed on the morning of the day prior to withdrawal of blood and infusion.

When the effect of pre-existing acidosis as a complicating factor was investigated, the animals were given 0.25 gm. of ammonium chloride per kilo body weight, administered as 0.9 per cent solution by stomach tube in three doses, *viz.* during the morning and evening of the day before bleeding and infusion and before anesthetization on the morning of withdrawal of blood.

On the day of hemorrhage and infusion the animals were anesthetized with 30 mg. of sodium pentobarbital per kilo body weight (0.44 cc. of 6.6 per cent solution) and shaved. A femoral cannula and urethral catheter were inserted. An initial sample of blood was taken and urine was collected for a 30 minute period for the determination of pre-hemorrhage urea clearance values. Each animal was then bled 50 cc. every 7 to 10 minutes until the total volume of blood drawn reached 50 cc. per kilo body weight. Plasma, or a solution of sodium chloride, of oxyhemoglobin, or of methemoglobin was then injected at the same rate at which the blood had been drawn and in the same volume. Ten minutes after the end of the infusion a sample of blood was drawn, urine was collected for a 30 minute period, and the urea clearance was determined. In some animals repeated urea clearances were measured during the ensuing 4 hour period during which the anesthesia was prolonged by additional sodium pentobarbital infusion. After the urea clearances were determined the femoral cannula was removed and the site of the operation sewn up, the catheter removed, and the animal returned to a metabolism cage.

As in the pre-hemorrhage period, fasting plasma urea nitrogen was determined daily each morning and the urine urea nitrogen output determined for the preceding 24 hours; 24 hour clearance values were calculated from these data. Clearance values and plasma urea nitrogen were followed till they had returned to the pre-hemorrhage level.

### RESULTS

#### *A. Experiments on Dogs without Pre-Induced Acidosis*

*Effect of Sodium Pentobarbital Anesthesia on the Urea Clearance and Acid-Base Balance.*—In eight normal dogs 24 hour urea clearances done for 5 consecutive days were averaged as the control clearance for each dog prior to the day of anesthesia, bleeding, and infusion. A urea clearance obtained in each of these dogs over a period of 30 to 60 minutes after injection of sodium pentobarbital showed no appreciable change in one animal, but in seven dogs the clearance fell to from 47 to 82 per cent of the control clearance and averaged 60 per cent of the control.

Sera of four normal dogs analyzed for  $\text{CO}_2$  and chloride before and 1 hour after injection of sodium pentobarbital showed no appreciable changes. The average  $\text{CO}_2$  was 24.5 mm per liter before and 25.5 one hour after anesthesia. The average chloride was 113.9 mm per liter before and 112.7 mm one hour after anesthesia.

*Controls without Replacement of Drawn Blood.*—Six dogs were bled without replacement. A period of about 12 hours' oliguria followed in each case, but all the dogs then resumed excretion, and all recovered. Blood urea nitrogen rose a few milligrams per 100 cc. during the oliguria, but did not usually exceed

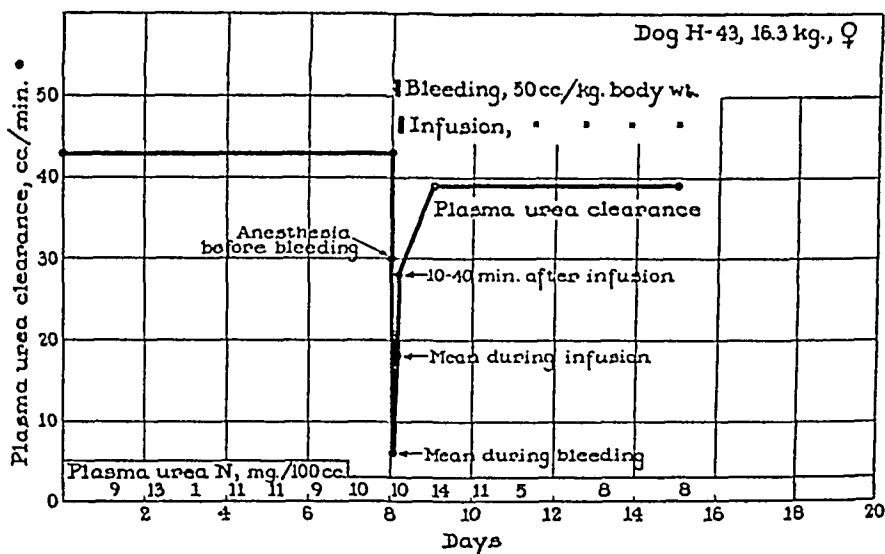


FIG. 1. Hemorrhage with plasma infusion.

20 mg. During the next day blood urea nitrogen and urea clearance had returned to approximately pre-hemorrhage level.

*Replacement of Blood by 0.9 Per Cent NaCl Solution.*—Two dogs were bled, and the withdrawn blood replaced with an equal volume of 0.9 per cent NaCl solution. The post-hemorrhagic oliguria was relieved by the injection. Both animals recovered and showed no significant elevation of blood urea nitrogen. Urea clearances were slightly depressed during the remainder of the day but returned to pre-hemorrhage levels on the following day.

Plasma  $\text{CO}_2$  in these two dogs fell following hemorrhage and infusion to 80 per cent of the initial values, but returned to normal on the following day. The fall in  $\text{CO}_2$  was balanced by an approximately equivalent rise in plasma chloride.

*Replacement of Blood by Plasma.*—Six dogs were used in this series. A typical result is shown in Fig. 1. The post-hemorrhagic oliguria is here shown by the low clearance before plasma infusion began. The oliguria was promptly re-



lieved by the plasma infusion. These animals all recovered without having shown any significant elevations of blood urea. Urea clearances during the hours after hemorrhage and plasma infusion were only slightly depressed, and on the following day were approximately the same as the pre-hemorrhage clearances.

It appears from these three sets of animals that the withdrawal of 50 cc. of blood per kilo body weight, whether replaced by plasma or NaCl solution, or not, does not affect kidney function for more than 24 hours.

An unexpected phenomenon following the plasma infusion was transitory edema, especially evident in swelling of the muzzle areas. The infusion increased the circulating plasma volume (although presumably not the total blood volume) to greater than pre-hemorrhage level; this increase may have been the cause of the edema.

#### *Replacement of Blood by 7.6 Per Cent Oxyhemoglobin Solution.—*

(a) *Effects on Renal Function.*—Four dogs were observed in this series. In each the post-hemorrhagic oliguria was at once relieved by the hemoglobin infusion. The urea clearance rose at once to pre-hemorrhage level (Figs. 2 and 3) and then fell again during subsequent hours. This secondary fall in clearance (which was not noted when the lost blood was replaced by plasma (Fig. 1)), appears to indicate specific, though temporary, damage to the kidney. In two of the four dogs there followed during the next day a rise of clearance to nearly pre-hemorrhage level (example, Fig. 2). Plasma urea nitrogen rose only a few milligrams. In the other two dogs, however, a number of days were required for complete recovery (Fig. 3), and plasma urea nitrogen rose higher (to 44 mg. in the dog of Fig. 3 and to 70 mg. in the other dog, not shown in the figures).

The edema noted after plasma infusions was not observed after the infusions of hemoglobin solution.

(b) *The Fate of Injected Oxyhemoglobin.*—The maximal concentration of hemoglobin reached in the plasma was approximately 5 gm. per 100 cc., observed immediately after finishing the infusion. The "half-life" of the oxyhemoglobin in the circulating plasma was about 8 hours; i.e., whatever hemoglobin concentration was present at a given moment in the plasma was reduced to about half in the next 8 hours. This is illustrated in Fig. 4, where the hemoglobin remaining in the plasma at various times after injection is expressed as a fraction of the maximal hemoglobin concentration obtained immediately after infusion. After 48 hours traces of pigment were still visible; in 72 hours the plasma was clear.

In the infused oxyhemoglobin solutions 3 or 4 per cent of the total pigment was methemoglobin, and this proportion was present in the plasma pigment at the end of the infusion, making a plasma concentration of about 0.2 gm. of

methemoglobin per 100 cc., total plasma hemoglobin being about 5 gm. per 100 cc. While the active hemoglobin disappeared rapidly, the methemoglobin

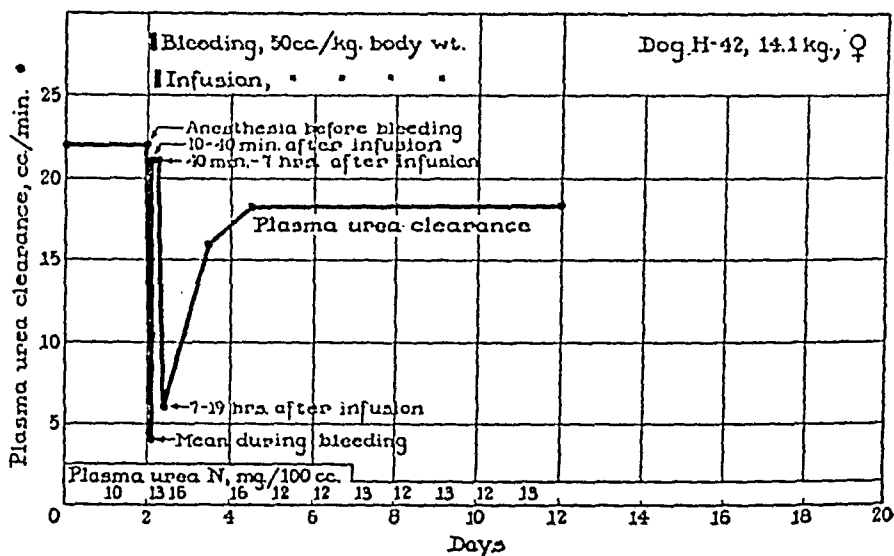


FIG. 2. Hemorrhage with oxyhemoglobin infusion.

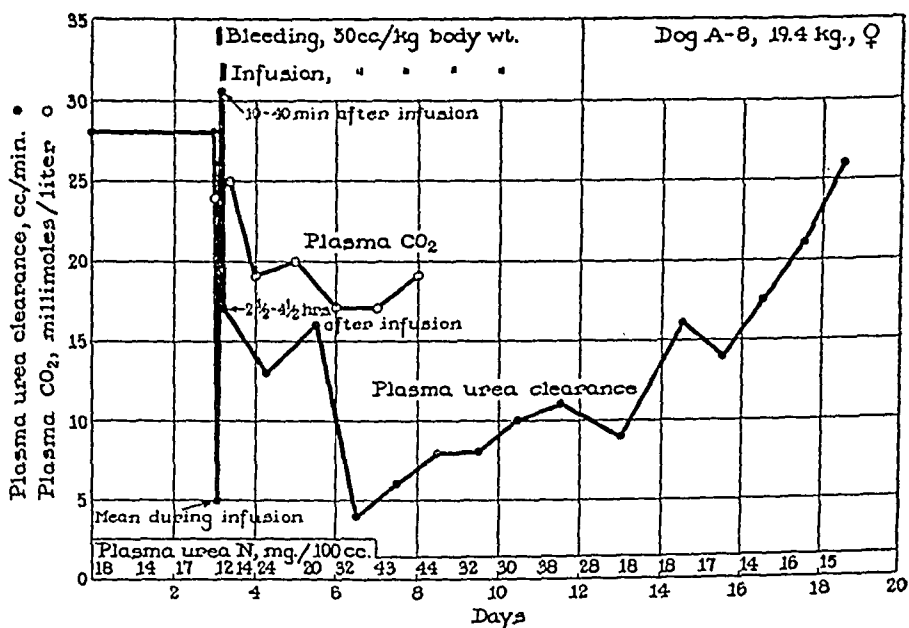


FIG. 3. Hemorrhage with oxyhemoglobin infusion.

concentration was approximately maintained for several hours, during which presumably a slow transformation of active hemoglobin into methemoglobin occurred, balanced by an equal rate of removal of methemoglobin from the

plasma. Later the methemoglobin diminished, and disappeared with the oxyhemoglobin.

Hemoglobin began to appear in the urine within about 15 minutes after the infusion was started. Altogether 30 to 40 per cent of the hemoglobin infused

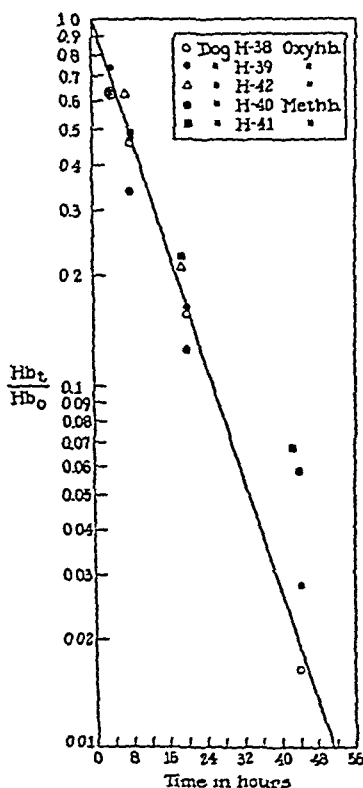


FIG. 4. Rate of disappearance of infused hemoglobin from plasma.  $Hb_0$  = maximal Hb concentration observed in plasma immediately after infusion.  $Hb_t$  = Hb concentration  $t$  minutes after end of infusion. The points are from five different experiments, three with oxyhemoglobin, two with methemoglobin. Disappearance rate was about the same with both types of Hb.

was excreted in the urine. The remainder was presumably taken up by the animal's tissues.

#### *Replacement of Blood by 7.6 Per Cent Methemoglobin Solution.—*

(a) *Effects on Renal Function.*—Two dogs were tested. The effects on renal function were not markedly different from the effects of oxyhemoglobin infusions. As in the dogs receiving oxyhemoglobin, the post-hemorrhagic oliguria, observed in the dogs receiving no replacement fluid, was at once relieved. Some

elevation of blood urea nitrogen and some depression of clearance values were observed; the plasma urea nitrogen rose for the first 3 days to maximum values of 54 and 69 mg. per 100 cc. and thereafter dropped rapidly to normal levels within the next 3 days. The urea clearance values remained somewhat depressed during the time of elevated plasma urea nitrogen and thereafter rose to normal levels. The findings in one of these animals are illustrated in Fig. 5. The further course of these animals was uneventful.

(b) *Fate of Injected Methemoglobin.*—The rate of disappearance of the methemoglobin from the plasma was practically the same as that of oxyhemoglobin, the half-life being about 8 hours, as shown in Fig. 4.

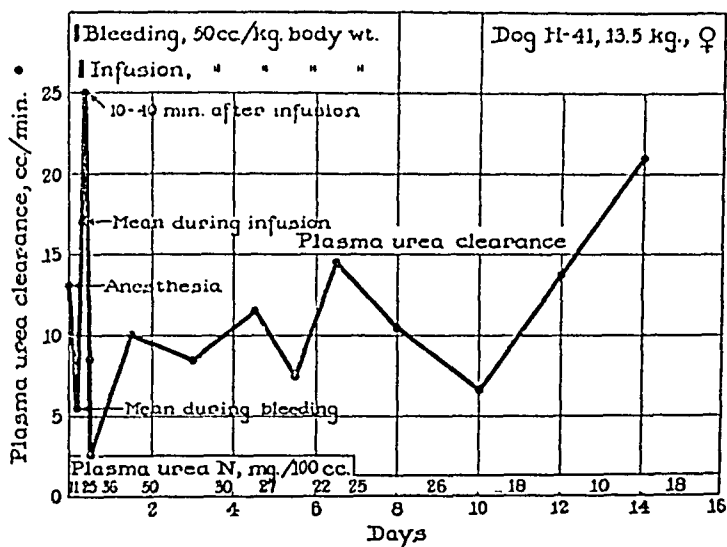


FIG. 5. Hemorrhage with methemoglobin infusion.

Transformation of methemoglobin to active hemoglobin (capable of binding  $O_2$  and CO) occurred much more rapidly than the reverse reaction noted after oxyhemoglobin infusions. Forty minutes after the end of an infusion about 40 per cent of the pigment present in the plasma was in the form of active hemoglobin. As the concentration of total hemoglobin in the plasma fell, the proportion in the form of active hemoglobin continued to rise, reaching 60 to 65 per cent 8 hours after the end of the infusion, and remaining there as long as enough pigment remained to be accurately measured.

In the urine about the same proportion of the injected pigment was excreted (30 to 40 per cent), as after infusion of oxyhemoglobin. In a urine which was analyzed for methemoglobin and total hemoglobin the ratio was found to be practically the same as that in the plasma during the excretion.

#### B. Experiments on Dogs with Acidosis Induced by Administration of $NH_4Cl$ before Hemorrhage

*Controls without Replacement of Drawn Blood.*—Hemorrhage itself always

caused a fall in plasma  $\text{CO}_2$  (e.g. Fig. 3). The somewhat greater degree of acidosis reached when  $\text{NH}_4\text{Cl}$  had been previously administered appeared to be without significant influence on the renal effects of the hemorrhage. There was the same transitory oliguria and period of depression of urea clearance, followed by return to normal clearance within 24 hours, as shown in Fig. 6.

*Replacement of Blood by Plasma.*—One acidotic dog was observed in which the drawn blood was replaced by an equal volume of dog plasma. The degree of acidosis was similar to that of the animal in Fig. 6, plasma  $\text{CO}_2$  being reduced

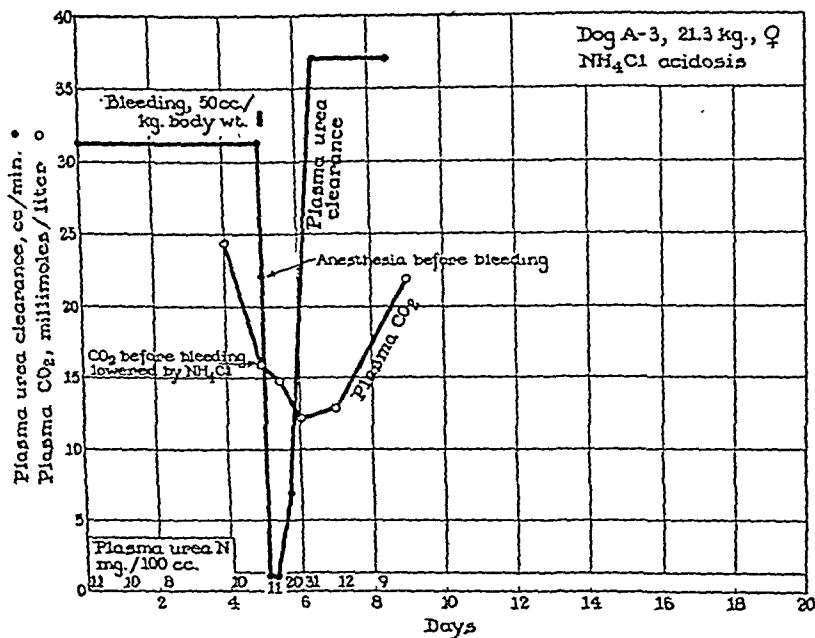


FIG. 6. Hemorrhage with no therapy.

from 25 to 17 millimoles per liter by the ammonium chloride, falling to 13 after hemorrhage, and returning to normal on the following day. The post-hemorrhagic oliguria was promptly relieved by the administration of plasma, urea clearance values returned to normal on the same day and remained normal thereafter. The plasma urea nitrogen never deviated from low normal values, 6 to 12 mg. per 100 cc.

*Replacement of Blood by 7.1 Per Cent Oxyhemoglobin.*—One animal, Fig. 7, was investigated. Comparison with Fig. 3 indicates but little influence of the acidosis on the renal effect of the replacement.

#### SUMMARY

Dogs were bled 50 cc. per kilo body weight and the blood withdrawn was re-

placed by equal volumes of 0.9 per cent NaCl solution, plasma, or 7 per cent oxyhemoglobin or methemoglobin solution.

Control dogs in which the withdrawn blood was not replaced by another fluid showed anuria or oliguria and depressed urea clearance for several hours after bleeding, but renal function returned to normal the next day.

Administration of 0.9 per cent NaCl solution or plasma promptly relieved the post-hemorrhagic oliguria and accelerated return of urea clearance values to normal.

Administration of oxyhemoglobin solution promptly relieved the post-hemorrhagic oliguria, but in some cases was followed by a period of 3 to 5 days in

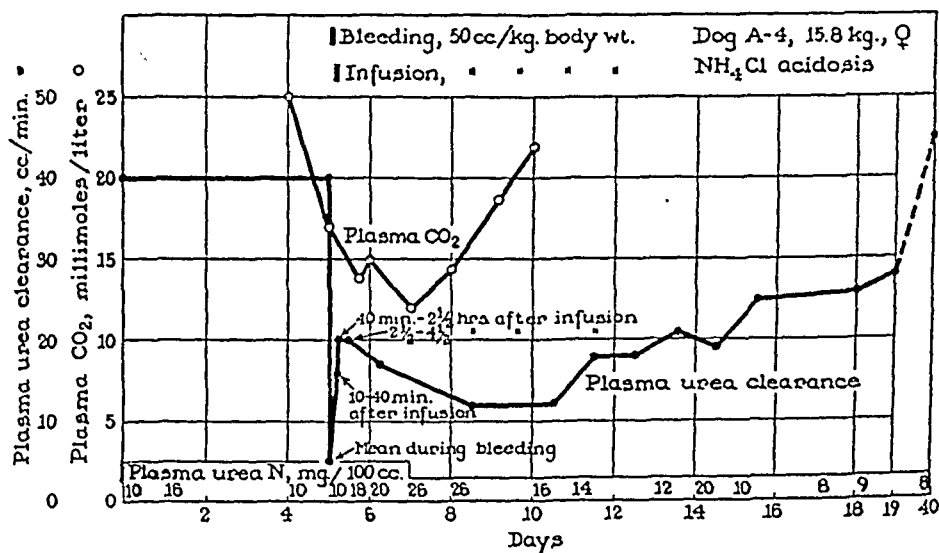


FIG. 7. Hemorrhage with oxyhemoglobin infusion.

which the urea clearance values were depressed to approximately 25 per cent of normal with an accompanying moderate elevation of plasma urea nitrogen. Return to normal clearance ensued during the next 5 or 6 days. The animals gave no signs of renal failure.

When pre-hemorrhagic acidosis was induced by the administration of ammonium chloride (plasma CO<sub>2</sub> reduced from the normal 25 to about 17 millimoles per liter) the renal behavior after replacement of blood by plasma or hemoglobin solution was essentially the same as in dogs not treated with ammonium chloride. However, since hemorrhage itself caused acidosis nearly as severe as that produced by NH<sub>4</sub>Cl the results do not exclude acidosis as a factor in the effects of all the experiments.

After infusion of either hemoglobin or methemoglobin the concentration of total hemoglobin in the plasma fell off at such a rate that any given concentration was reduced by about 50 per cent in 8 hours. A small amount of pigment

remained at the end of 48 hours in the circulating plasma but none was detectable at the end of 72 hours.

When methemoglobin was introduced into the circulation, it was rapidly converted into active hemoglobin. After injecting a 7.6 per cent solution of methemoglobin (99 per cent methemoglobin), 41 per cent of the pigment in the circulating plasma was found to be active hemoglobin 35 minutes after the injection was completed.

No significant difference was noted between infused oxyhemoglobin and methemoglobin, either in effects on renal function or in rates of disappearance from circulation and excretion in the urine.

### CONCLUSION

The immediate effects of treating hemorrhagic shock in dogs by replacing lost blood with 7 per cent hemoglobin solution were favorable, both on renal function and on general condition. However, subsequent transitory depression of the urea clearance for several days, shown by some of the treated animals, but not by untreated bled controls, indicates sufficient possibility of renal damage by the hemoglobin solution to prevent its recommendation at present as a blood substitute.

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# STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS

## X. ANTIBODIES INDUCING IMMEDIATE-TYPE SKIN REACTIONS\*

By MERRILL W. CHASE, Ph.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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In experiments on the sensitization of animals to certain simple chemical compounds, antibodies which induce passive anaphylaxis have been demonstrated in the serum (1). It has since been found possible to elicit reactions of the "early type", simulating the Prausnitz-Küstner reaction, in normal guinea pigs at skin sites prepared with the serum of sensitized animals.<sup>1</sup> The effect has been obtained with acyl chlorides, acid anhydrides, picryl chloride, and 2:4 dinitrochlorobenzene. Experiments of this type are described in the present paper together with related observations on reactions occurring in the skin of normal guinea pigs as a result of interaction between antibodies and common antigens.

### EXPERIMENTAL

*Sensitization of Animals and Production of Antisera.*—For obtaining sera of satisfactory potency, it was necessary with most animals to extend the sensitizing injections over 5 to 12 weeks or more, with intervening rest periods. The simple chemicals dissolved in olive oil or in corn oil were injected intradermally into male albino guinea pigs,<sup>2</sup> each injection consisting of 0.05 cc. of the vehicle containing 1/20 mg. of the incitant in most cases (*o*-chlorobenzoyl chloride, citraconic anhydride, phthalyl chloride, 2:4 dinitrochlorobenzene), but 1/80 mg. of picryl chloride, and 1/5 mg. of phthalic anhydride. With the last substance, a concentrated solution in dioxane was added to olive oil (*cf.* Jacobs (6)). Once sensitivity had become established, the reactions developing upon subsequent injections were examined early (between 30 minutes and 2 hours) as well as the next morning, since there is evidence for two maxima corresponding respectively to the early and the delayed-type reactions (7, 9). Although the presence of effective concentrations of transfer antibodies could be determined only by trial bleedings and by testing the serum as described below, the occurrence of intense early reactions (with at times subsidiary reactions of the early type around old injection sites (*cf.* 6-8)) seemed to give the best indication as to probability of a high serum antibody level. It may be men-

\* The work here presented was largely done in association with the late Dr. Karl Landsteiner, and was cited as item No. 344 in the latter's bibliography (2).

<sup>1</sup> These have been reported briefly in reference 3; similar effects with antiprotein sera have also been found (4).

<sup>2</sup> Of the several stocks of albino guinea pigs employed, progeny from the colony established genetically as highly susceptible to experimental sensitization with 2:4 dinitrochlorobenzene (5), and subsequently pen-inbred, may have been somewhat superior in regularity of production of transfer sera.

tioned that the choice of olive oil or corn oil as vehicle for the chemical substances either in sensitizing or in eliciting reactions was shown to be immaterial for the results.

To describe a typical experience with ortho-chlorobenzoyl chloride:—

One lot of 13 male albino guinea pigs was injected intradermally with 1/20 mg. of the chemical dissolved in 0.05 cc. corn oil on 10 occasions over a period of 3 months, arranged in 4 courses with intervening rest periods of 16 or 17 days—on days 1, 4, 8, 12; 29, 36, 43; 59, 65, 73; 89. Twenty-eight trial bleedings made during this time revealed the transfer antibody in 11 of the 13 guinea pigs; the best 4 animals were exsanguinated for serum—1 on the 46th day, another on the 81st day, 2 on the 97th day. The various bleedings were tested in the skin of normal animals as described later; the test dose of *o*-chlorobenzoyl chloride was 1 to 2 mg. given by the subcutaneous route. Reactions developing in 45 to 60 minutes with this substance were considered satisfactory. In a similar but more prolonged experiment with citraconic anhydride, a total of 11 injections was made over 19 weeks, and every guinea pig in the lot of 10 eventually developed a good content of transfer antibody. In all, inclusive of series treated over shorter periods of time, 44 satisfactory transfer sera were secured from a total of 80 animals sensitized to citraconic anhydride. In the case of this substance, sites prepared on normal recipients with such sera commonly reacted in 3 to 10 minutes.

The sera reacting with picryl chloride, developed by repeated intradermal injections of olive oil solutions, were nearly always less potent than those prepared in the same way to acid anhydrides and acyl chlorides (e.g., transfer sites in normal recipients reacted in 40 to 60 minutes after subcutaneous injection of picryl guinea pig serum) and were encountered not too frequently. For instance, in the course of sensitizing injections on 13 guinea pigs, spaced approximately as described above, a total of 10 trial bleedings between the 37th and 72nd days demonstrated the antibody in 9 of the animals. Similar sera but of much higher activity were encountered almost regularly in animals which, between 4 and 7 weeks previously, had received intramuscularly an emulsion of picryl guinea pig serum, or picryl guinea pig stromata, along with paraffin oil and killed tubercle bacilli.<sup>3</sup>

With 2:4 dinitrochlorobenzene, transfer antibodies were obtained following intracutaneous injections (10 injections of 1/20 or 1/40 mg. of the chemical in 0.05 cc. olive oil over a period of 9 weeks), although the concentration of antibody in the sera was often low. Even in effecting sensitization by means of painting the skin ten times with a 2 per cent solution in alcohol, these antibodies were clearly, although feebly, detectable now and then. Another method which has yielded more active sera consisted of several intracutaneous injections of 1/50 mg. of the incitant dissolved in paraffin oil, to which killed tubercle bacilli had been added. After injections on five occasions over an 18 day period, 7 positive sera were found among 18 guinea pigs bled between the 28th and 40th days.

Because transfer reactions with drug hypersensitivity sera were so readily elicited by the corresponding protein conjugates, antiprotein sera were prepared in guinea pigs (4). (The analogous work of Ramsdell (13), not then known to us, will be described later.) Horse serum (0.1 cc. of a 1:5 dilution) was injected intradermally, approximately in the sequence

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<sup>3</sup> These emulsions were made with the aid of commercial preparations derived from lanolin, namely Aquaphor (Duke Laboratories) or Falba (Pfaltz and Bauer) and contained 10 mg. conjugate and 0.5 mg. dried tubercle bacilli in 1 cc., the amount injected into each animal, distributed between 4 or 5 sites. Such emulsions were used in preference to injecting the aqueous and oily phases separately as we did originally in sensitizing guinea pigs to picryl chloride by means of a protein conjugate and a suspension of killed tubercle bacilli in paraffin oil (10). The preparation of Aquaphor emulsions incorporating dead tubercle bacilli in paraffin oil was devised by Freund as a method which confers substantial adjuvant effect on several antigenic materials (11, 12; cf. 4).

shown above for the first two courses with *o*-chlorobenzoyl chloride; on the 48th day, 1 week after the last injection, the sera of all 8 guinea pigs were found to give a transfer effect. A second method, usually giving more active sera, was the procedure developed by Freund (11) namely a single injection into the muscles of the neck of an emulsion of horse serum, Aquaphor, and killed tubercle bacilli in paraffin oil; the animals were bled 4 or preferably 6 weeks or longer afterwards.

In view of the results obtained with horse serum, the preparation of anti-ragweed sera was undertaken. This proved possible (4) by intracutaneous injections of alumina flocs of pollen extract or, apparently less regularly, by subcutaneous injection of defatted pollen incorporated in an Aquaphor emulsion with paraffin oil and dead tubercle bacilli. The alum precipitates were made from a "7 per cent" extract of defatted low ragweed (*Ambrosia elatior*) pollen in Coca's alkaline fluid prepared without phenol, by adding 1/9 volume of 10 per cent potassium alum solution and sufficient  $\text{N}/10$  NaOH to neutralize the mixture; 0.1 cc. portions of such freshly prepared suspensions were injected into the skin twice a week for 3 weeks; then further injections were given after a month's rest, and the sera were taken between the 9th and 12th weeks. Transfer sera against ragweed, of apparently higher titer, have since been obtained in guinea pigs by Kulka and Hirsch (14).

Trial bleedings were made by heart puncture 1 to 3 days, rarely later, following a routine sensitizing injection and were tested in normal guinea pigs as described later. The donors chosen were exsanguinated between the 2nd and 10th days after the preceding sensitizing injection, commonly around the 5th day. The sera were stored in the refrigerator at  $4-7^{\circ}\text{C}$ . without addition of preservative.

**Sensitizing Chemicals.**—Ortho-chlorobenzoyl chloride and citraconic anhydride, Eastman preparations, were redistilled *in vacuo*. Phthalyl chloride, a Kahlbaum preparation, was purified by fractional freezing. Injection of these three substances was made with solutions in oil prepared immediately beforehand. Phthalic anhydride was a commercial preparation (Eastman). Picryl chloride was twice recrystallized from a benzene-alcohol mixture, while 2:4 dinitrochlorobenzene was recrystallized from alcohol.

**Protein Conjugates.**—For the preparation of *o*-chlorobenzoyl and phthalyl conjugates, 10 cc. of guinea pig serum was treated with 0.7 cc. of  $\text{N Na}_2\text{CO}_3$  and shaken for several minutes with 0.5 cc. of chloroform containing 0.05 cc. of *o*-chlorobenzoyl chloride or phthalyl chloride; a small additional amount of chloroform was later added and the shaking continued for a time. The aqueous solution was separated from the chloroform layer and the conjugated protein brought to flocculation by careful dropwise addition of  $\text{N HCl}$ . Where the amount of precipitate was rather heavy, several volumes of saline were added. After centrifuging and discarding of the supernatant solution, the precipitate was dissolved in a small amount of saline with addition of  $\text{N NaOH}$  to neutrality, any insoluble residue being removed. The dry weight was determined upon a portion precipitated with alcohol.

"Citraconyl guinea pig serum" was made by shaking 0.05 cc. of citraconic anhydride with 10 cc. serum for several minutes, any precipitate forming meanwhile being dissolved by partial neutralization with  $\text{N NaOH}$ . The mixture was precipitated by addition of  $\text{N HCl}$ , the conjugated protein separated by centrifugation and washed with saline; it was then dissolved in saline by adding  $\text{N NaOH}$  to pH 7.0.

The preparation of "picryl guinea pig serum" followed the method already described (1). Picryl casein was made similarly, with a 7 per cent solution of Hammarsten's casein dissolved with sodium carbonate instead of serum, but precipitation with alcohol was omitted. In analogous manner, starting with dinitrofluorobenzene, "dinitrophenyl guinea pig serum" was prepared (cf. 1), here likewise with omission of the alcohol precipitation.

**Preparation of Skin Sites; Recipient (Acceptor) Animals.**—Male albino guinea pigs weighing between 350 and 450 gm. served as recipients. Injections of guinea pig sera (0.15 cc. or less)

were made intradermally on the back between the loose skin over the pad of fat in the nuchal region and the skin over the pelvic girdle, and distant by 22 to 30 mm. from the middorsal line. In the routine testing of sera, as many as 8 sites were prepared and tested simultaneously on one animal: this must be done with discretion when it is to be expected that 5 or 6 sites may react, not only because strong reactions may spread and become confluent but also because a portion of the antibody introduced at each site escapes into the circulation and contributes to a systemic reactivity. When multiple like sites react, the extent of the individual reactions is apt to be less than when the sera are tested singly; a similar situation was encountered by Fischel and Kabat (15) in studying the transferred Arthus phenomenon in the rabbit.

With a given serum, the development and extent of the transfer reactions vary with the individual recipient and with age (weight). In addition, there may be differences in strains, since of the 2 stocks of guinea pigs chiefly employed animals of the one described in footnote 2 have given more intense reactions. Male albino guinea pigs weighing between 350 and 450 gm. have been used almost exclusively in the present experiments, but animals of 200 to 275 gm. weight of either sex give larger and more striking reactions under the same conditions of test; conversely, guinea pigs weighing 500 gm. and over have rarely proved satisfactory, exhibiting erythema and edema only meagerly. As mentioned, in any weight group and sex, individual variation occurs, and although only a few have failed to give indubitable responses, some animals have proved to be poor "acceptors," giving responses of feeble intensity and small diameter. When in a small experiment histamine dihydrochloride (0.05 cc. of 1:1200 dilution calculated as the free base) was injected into the skin of two selected classes of animals, namely individuals which had given large and prominent transfer reactions and those which had not, it turned out that members of the former group were all characterized in 10 to 15 minutes by distinguishably larger, colorless swellings; but the method did not reveal sufficient gradations to suggest its use for practicable preselection of recipients. To recognize individual differences among acceptors, it has been our practice in routine work to include a skin site prepared with a standard serum of the same sort as or, preferably, one of different specificity from that under test; in the latter case, an evaluation of the individual recipient may be made at some subsequent time without influencing the reaction under study.

*Testing of Antisera.*—Testing was usually done 1 or 2 days after the preparatory injections, as soon as the sites were essentially normal in appearance. (With sterile guinea pig sera, toxic reactions were seldom encountered.) The specific reactions, developing in the prepared sites and spreading peripherally, with erythema and edema, could be elicited either by local application of suitable test materials or by injection at a remote point. Where the simple chemical incitant is readily reactive, for instance with proteins, injection of amounts of about 1 mg. (citraconic anhydride, phthalyl chloride) or 2 mg. (phthalic anhydride, *o*-chlorobenzoyl chloride) in a bland oil beneath the skin of the abdomen commonly produced a clear reaction within 3 to 45 minutes. In the same way, picryl chloride (1 mg.), which, chemically, reacts more slowly, led to observable reactions after 45 to 200 minutes; subcutaneous administration of the still less reactive 2:4 dinitrochlorobenzene was not effective (see footnote 5). Upon local injections of oil solutions into the prepared sites (in which case low concentrations are necessary to avoid toxic effects and the volume must be small), reactions ensued but these tended to be small and blurred, probably because of slow release of the chemical substance. With some substances, however, scratch tests made through dioxane solutions across the prepared sites<sup>4</sup> could be used to advantage (citraconic anhydride, phthalic anhydride). With citraconic anhydride, scratches were made through a drop of 10 to 25 per cent solution in dioxane placed on the skin; similarly adequate concentrations of most other substances had some primary toxicity

<sup>4</sup> Or scratches may be made elsewhere on the normal skin, with the consequence that the latent period is somewhat longer before reaction at the serum site starts.

for skin, so that the application was best confined to the scratch line. For this purpose, scratches were made with a No. 26 hypodermic needle (attached to a loaded syringe) having a very small pendent droplet of the solution on the needle tip. With phthalic anhydride, a scratch test with a 25 per cent solution in dioxane was used, and this served well for testing either phthalic anhydride (*cf.* 16) or phthalyl chloride sera. In some other cases, scratch tests across prepared specific sites also evoked reactions (40 per cent picryl chloride in dioxane, initiating a reaction in 50 to 120 minutes, and 15 per cent phthalyl chloride in olive oil, producing a reaction of small size that started within 30 minutes). The reaction is readily induced when soluble protein conjugates are substituted for the free chemical: local injections of rather small quantities may be made directly into the prepared area, or the conjugate in larger amount (1 to 50 mg.) may be injected subcutaneously at a remote site or intraperitoneally or intravenously. Central wheals are most evident following the local injections of conjugates. When sites have been prepared with sera of low antibody content, the tests have seemed to be best made by the subcutaneous injection of fairly large amounts of protein conjugate (say 50 to 75 mg.). With sera to 2:4 dinitrochlorobenzene, the antibody has so far been satisfactorily demonstrable only with the corresponding dinitrophenyl protein conjugate.

*Characteristics of the Reaction.*—After the preparatory injection, an interval of 7 to 10 hours is necessary for the skin site to become typically reactive (see also 17). Tested prior to this time, the serum site may show at best some transient slight degree of increased volume, the same effect as may be noted when a conjugate (or protein antigen) and the corresponding antiserum are injected as a mixture; in both of these cases, the potential reactivity of the site is "discharged" and no reaction will ensue at a later retest (*cf.* 18). (In the rabbit, the situation appears to be different: Ramsdell (13) found the rabbit ear to respond as soon as antiserum was deposited in the tissue, and Fischel and Kabat in a recent study (15) injected antibody and antigen into the same area of rabbit skin at an interval of 30 minutes in order to develop the delayed response characteristic of the Arthus phenomenon.)

After the skin site has become capable of reaction there is a latent period—typically 3 to 30 minutes—between administration of the substance and the first appearance of a perceptible reaction. Of the various factors which influence the length of this latent period, apart from the question of the nature of the chemical substance, the foremost are the concentration of antibody in the antiserum, and the adequacy of the method of testing as to route and amount of substance; of lesser bearing is individual variability among recipient animals. Variations in the latent period arising from the different reactivities of the various chemicals may be nullified by testing with preformed conjugates of the chemical substances with proteins, and the sera can then be compared under equivalent conditions. That the latent period may reflect the strength of the antiserum was observed in consecutive trial bleedings from animals undergoing active sensitization and in several of the experiments in which various dilutions of one antiserum were used to prepare skin sites (see p. 504).

Almost independently of the manner of testing, which influences chiefly the amount of reactive material available and the rapidity of absorption, and thereby the latent period before the reaction starts, there usually appear first discrete dots of color, rash-like, over an area of 1 or 2 cm., then additional pinkish patches, and confluence, all within 5 minutes (*cf.* 4); the onset of edema is apparent even then. In the most striking cases, the effect starts as an even, bluish (cyanotic) discoloration with transient but prominent erythematous streaks across the blue area; hyperemia and edema start almost at once at the periphery, and the pinkish color extends slowly into the bluish center which, incapable of developing edema for some minutes, probably because of intense vasoconstriction, forms an umbilicate center in the reaction area. In either case, the erythema spreads from the central area peripherally and pseudopodially, rapidly at first, followed more slowly by progressive edema which gradually replaces the erythema. The erythema may be maximal 10 to 20 minutes after onset of the reaction, while the

greatest diameter and swelling of the local area are attained still later. The area involved in a reaction varies with the strength of the serum and with the particular animal; as mentioned above, when one animal has several like sites reacting at the same time, the areas are usually smaller. Reactions as large as  $97 \times 50$  mm., involving practically the entire flank, have been encountered, but even with large reactions it is rare that the middorsal line is crossed. Except for occasional faint recrudescence of erythema a few hours later, which may be confined to the central part of the area previously reacting, there is typically no further change, the edema receding in its turn and the skin appearing normal on the next day; following intense reactions, brownish pigmentation may be visible for a day or so. (With antisera against horse serum, and sometimes with antisera against *o*-chlorobenzoyl chloride, there may be definite effects still present on the following day.)

When a typical, homologous reaction has once been induced, the site will not respond to a second testing, reflecting depletion of antibody. In the case of intense reactions, there may be a second factor, to be referred to later; the skin area does not fully recover its capacity to react, even to a different antigen-antibody system, for perhaps 6 to 8 days. When reactions are partial because of the use of insufficient antigen or allergenic chemical, a prompt retesting with adequate material may call forth a second response, but the sites tend to become discharged (*cf.* Table VIII, test and retest of recipient 3). A few instances have been encountered in which sites have been discharged without exhibiting visible reaction, the specific substance corresponding to the antibody having been given subcutaneously in small amount on the day prior to the adequate test; on the other hand, the situation shown in test and retest of recipient 4, Table VIII, is met with more often. These effects within a localized area, it will be recognized, have counterparts in the desensitization of animals (or organs) in the passively induced anaphylactic state.

If no reaction is induced, the area remains responsive for 5 or 6 days, sometimes rather well so for 3 weeks or more. While the length of fixation varies with the particular serum, probably reflecting its antibody content, the capacity of the local site to react is found to lessen, gradually, after the first few days.

*Exhaustion of Cutaneous Reactivity by Local Reactions.*—It has been stated that an intense reaction may result in a temporary exhaustion of the capacity of a skin area to respond to re-stimulation. This exhaustion is perhaps best shown by inducing and charting the area of rather large transfer reactions on several animals. At various times thereafter on different animals, starting 2 days later, small amounts of an antiserum of another specificity may be injected along the margin and in the center of one of the areas that reacted previously. When this antibody is brought to reaction 1 day after its injection, sites at the periphery of the old area usually show a distinct crescent shape, with the concave side determined by the border of the initial area of reaction; thin, pincer-like projections of erythema may then spread into the area to complete a circular outline, and the extended crescent may slowly fill in, thereby producing a disc-like reaction, the newer segment not infrequently having lesser color and elevation. Sites made in the very center of the old area show substantially greater inhibition, and on the 3rd or 4th day after the primary reaction may show no more than fine dots of erythema over an area of perhaps  $20 \times 20$  mm. This undoubtedly reflects the greater intensity of reaction in the center of an area (at the site of antibody deposition), just as does the central vasoconstriction seen at times in the primary reaction, or central whealing in human Prausnitz-Küstner skin tests. By the 7th day, the old reaction area is nearly normal, and at the 9th day is indistinguishable from other areas of skin.

A more widespread, but less complete, exhaustion of the skin's capacity to react has been noted as well. When several successive reactions in different skin areas are to be developed at short intervals, as in the type of experiment shown in Table VI, but not illustrated therein, the last reaction is apt to be feebler in color and less sharply demarcated than its predecessors.

TABLE I\*

*Passive Local Sensitization of Normal Skin by Sera from Sensitized Guinea Pigs*

Sera injected intracutaneously on the back in amounts of 0.15 cc. Test substances, dissolved in 0.25 cc. corn oil, injected 2 days later under the skin of the abdomen.

Recip- ient	Test injections		Sera used in preparing skin sites			
	Time	Substance	Anti-o-chlorobenzoyl chloride	Anti-citraconic anhydride	Normal guinea pig serum 1	Normal guinea pig serum 2
No. 1	0	o-Chloroben- zoyl chloride 2 mg.	i = 10' 26 X 24 ++, sl. el., 19' 53 X 40 +, m. swol., 67' soft,	Neg.	Neg.	Neg.
	4½	Citraconic an- hydride 1 mg.	No change	i = 7' 60 X 54 +, el., very 20' soft,	Neg.	Neg.
2	0	Citraconic an- hydride 1 mg.	Neg.	i = 4' 28 X 28 ++++, well 13' el., 22' 29 X 28 +, m. el., 70' Color receded,	Neg.	Neg.
	4½	o-Chloroben- zoyl chloride 2 mg.	i = 14' 25 X 25 +, sl. el., 30' Same, well el., 55' Color receding, 105'	No change	Neg.	Neg.
3	0	Citraconic an- hydride 1 mg.	Neg.	i = 4' 30 X 25 ++, el., 14' 36 X 29 +, el., 24' Color receded, 70'	Neg.	Neg.
	4½	o-Chloroben- zoyl chloride 2 mg.	i = 17' 24 X 22 +, el., 30' 29 X 23 +, v. m. el., 55' Color receded, 105'	No change	Neg.	Neg.

\* In this and other tables, the interval (in minutes) between administration of the chemical and appearance of a reaction is designated by "i." The other entries show first the reaction area (cross diameters given in millimeters), then the degree of erythema ( $\pm$  to ++++), finally the extent of edema. The key to abbreviated words is: sl. el., slightly elevated; v. m. el., very markedly elevated; swol., swollen; al. cls., almost colorless; ps., pseudopodial; tr., trace. Estimation of the edema seemed adequate, for it proved best to examine the area by very gentle palpation and to avoid folding of the skin.

*Transfer Effects with Compounds of High Reactivity*

Sera having a suitably high concentration of antibodies, obtained by injection of acyl chlorides and acid anhydrides, were injected in amounts of 0.1 to 0.15 cc. intracutaneously into the skin of the flank or back of normal guinea pigs. The skin sites so prepared reacted specifically in the manner described, when, on the following day, the corresponding incitant was injected locally into

the area or elsewhere, injections under the abdominal skin being used preferentially. The reactions could be elicited not only by the simple substances but also by protein conjugates of the respective chemical substances; e.g., "phthalyl guinea pig serum," and the like.

A representative experiment is shown in Table I. Four skin sites were prepared on the backs of normal guinea pigs by injection of sera, two being from untreated guinea pigs and the others from animals sensitized respectively to *o*-chlorobenzoyl chloride and citraconic anhydride. After the lapse of 2 days the two substances, dissolved in corn oil, were injected subcutaneously in turn at an interval of several hours. Reactions developed after latent periods of 4 to 17 minutes. It is seen that regardless of which substance was injected first only the corresponding serum site reacted. The second round of testing demonstrated that sites failing to respond to the prior test material had remained capable of reacting.

#### *Transfer Effects with Allergens of Lesser Chemical Reactivity*

Transfer antibodies were also produced by allergens of lesser chemical reactivity, even though with these substances sera of satisfactory potency were encountered less frequently. The reactions of sera obtained after courses of injections with picryl chloride are shown in Table II, in comparison with a serum obtained to *o*-chlorobenzoyl chloride. Here likewise, regardless of the order of injecting the test substances, the corresponding serum site alone reacted. It will be noted that the reactions induced by picryl chloride developed slowly and only after fairly long latent periods (70 to 170 minutes), in contrast to the earlier response to the more highly reactive substance *o*-chlorobenzoyl chloride. When, however, the testing was done with preformed conjugates (recipients 3 and 4), the site prepared with serum to picryl chloride reacted promptly and much like the site prepared with serum against *o*-chlorobenzoyl chloride. The difference in reaction times seen with recipients 1 and 2 was therefore not a reflection of significantly different concentrations of antibody in the two sera but rather of the chemical individuality of the simple allergenic substances under test. Indeed, with antisera produced by treatment with 2:4 dinitrochlorobenzene, which is chemically less reactive than picryl chloride (*cf.* 1), the simple substance itself gave no more than a much delayed, transient graying of a prepared site (10 hours after testing).<sup>5</sup> In contrast, the antibodies were readily and typically demonstrable by the use of conjugates, as in Table III.

<sup>5</sup> For this effect, the skin was painted with 6 drops of a 10 per cent solution in olive oil at some distance from the prepared site, since this concentration has a high primary toxicity for normal skin. The highly reactive substance 2:4 dinitrofluorobenzene injected directly into prepared sites may have neutralized the antibody in the very center of the prepared area, but not the peripheral zone.



TABLE II

*Passive Local Sensitization of Normal Skin by Sera from Sensitized Guinea Pigs*

Experiment as in Table I, except that picryl chloride was injected in 0.5 cc. corn oil and the soluble protein conjugates were given in 1 cc. saline.

Recip- ient	Test injections		Sera used in preparing skin sites		
	Time	Substance	Anti-picryl chloride, No. 1	Anti- <i>o</i> -chlorobenzoyl chloride	Normal guinea pig serum
No. 1	0	Picryl chloride 2 mg.	$i = 86'$ el. papules over $17 \times 12$ , $100'$ $22 \times 19$ $\pm$ , knobby, v. m. el., $120'$ $26 \times 26$ $++$ , enor. swol., umbilicate, $145'$ $31 \times 29$ $++$ , enor. swol., $192'$	Neg. " "	Neg. " "
	5	<i>o</i> -Chloroben- zoyl chloride 2 mg.	No change " "	$i = 22'$ $29 \times 24$ $++\pm$ , m. el., $40'$ $35 \times 29$ $++\pm$ , " " $100'$	Neg. "
2	0	<i>o</i> -Chloroben- zoyl chloride 2 mg.	Neg. "	$i = 18'$ $25 \times 19$ $++\pm$ , sl. el., $38'$ $27 \times 20$ $\pm$ , " " $133'$	Neg. "
	3	Picryl chloride 2 mg.	$i = 110'$ small scattered dots, $110'$ $22 \times 22$ large spots $++$ , $124'$ $33 \times 25$ $+$ , not el., $184'$ $53 \times 40$ $\pm$ , " " $290'$	No change " " " "	Neg. " "
3	0	Picryl casein 45 mg.	$i = 10'$ $45 \times 35$ $+$ (spotty), cls. center, $17 \times 15$ $10'$ $48 \times 48$ $++$ , cls. center $12$ , $13'$ Much faded, $50'$	Neg. " "	Neg. " "
4	0	<i>o</i> -Chloroben- zoyl guinea pig serum 50 mg.	Neg. "	$i = 10'$ $25 \times 20$ $+$ , sl. el., $5'$ $25 \times 22$ $++$ , v. m. el., $40'$	Neg. "
			Anti-picryl chloride, No. 1	Anti-picryl chloride, No. 2	Normal guinea pig serum
5	0	Picryl chloride 1 mg.	$i = 70'$ $37 \times 32$ $+$ , prac. confluent, el., $99'$ $70 \times 48$ $\pm$ , v. well el., $200'$	$i = 170'$ $36 \times 35$ $\pm$ , sl. el., $200'$ $50 \times 40$ cls., el., $290'$	Neg. " "

In this experiment, along with sites receiving the dinitrochlorobenzene serum, there have been included sites prepared with two guinea pig sera taken after a course of injections with phthalyl chloride. Upon testing with "2:4 dinitrophenyl guinea pig serum," the homologous site, it will be seen, reacted promptly and typically, while one of the phthalyl sera gave a delicate cross-reaction, coming to expression more promptly and definitely when the amount

TABLE III  
*Experiment as in Table II*

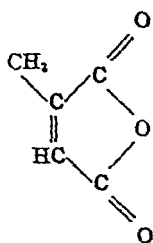
Recipient	Test injections		Sera used in preparing skin sites				Time	
			Anti-2:4 dinitrochlorobenzene	Anti-phthalyl chloride No. 1	Anti-phthalyl chloride No. 2	Normal guinea pig serum		
No.	hrs.							
1	0	Dinitrophenyl guinea pig serum 10 mg.	i = 12'					
			22 × 20 +, sl. cl.			Neg.	19'	
			28 × 24 +, cl.			"	40'	
			28 × 24 ±, "			"	120'	
			Neg.		Neg.	i = 168'		
						Delicate patchy erythema over 18 × 13	"	203'
					"	19 × 15 +, (mottled), not cl.	"	263'
24	Phthalylguinea pig serum 10 mg.	Neg.	i = 9'	i = 9'				
			23 × 21 ±, flat	25 × 25 ±, sl. cl.	Neg.	12'		
			26 × 26 +, sl. cl.	33 × 30 ±±, v. m. cl.	"	27'		
2	0	Dinitrophenyl guinea pig serum 50 mg.	i = 7'					
			31 × 29 +, m. swol.	Neg.		Neg.	16'	
			33 × 31 +, enor. swol.	"		"	35'	
				"		"	40'	
			Neg.			i = 30'		
						24 × 24 ±, delicate erythema, ps. margin, sl. cl.	"	
						26 × 26 +, sl. cl.	"	
24	Phthalylguinea pig serum 50 mg.	Neg.	i = 6'	i = 6'				
			30 × 30 ++++, m. cl.	40 × 40 ++++, m. cl.	Neg.	10'		
			50 × 35 ++, v. m. cl.	48 × 45 ++, v. m. cl.	"	20'		
			55 × 42 ±, swol.	55 × 50 ±, swol.	"	30'		

of conjugate was increased fivefold (recipient 2), and the other phthalyl serum did not respond to either test dose. Injection of "phthalyl guinea pig serum" on the following day caused both of the homologous test sites to respond within 10 minutes, and without evident diminution in the case of the serum which had exhibited the cross-reaction. Despite the partial crossing, which will be discussed later, the reactions exhibited, therefore, the attributes of serological specificity, in that the homologous antigen-antibody reaction was the greater.

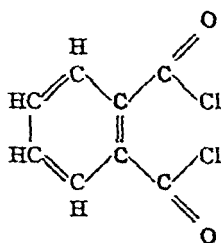
#### *Specificity of the Transfer Reactions*

Reactions of absolute specificity were presented in Tables I and II. In several cases cross-reactions of varying magnitude were seen, paralleling those

encountered in actively sensitized animals. Observed instances and some expectancies are recorded in Table IV. The cross-reactions can be ascribed largely to a substantial degree of configurational identity (see formulae), particularly among the products formed upon conjugation of the respective substances (e.g., phthalic anhydride, phthalyl chloride, citraconic anhydride) with proteins. These cross-reactions were not inconsiderable but were weaker than the homologous ones and on the second injection the reactions were specific, an effect comparable to a serum made specific by absorption with a heterologous antigen. Such a case is illustrated in Table V, which shows an outstanding instance of cross-reactivity, almost surely reflecting similar chemical configurations, namely that between citraconic anhydride and phthalyl chloride (or similarly phthalic anhydride).



Citraconic anhydride



Phthalyl chloride

With respect to cross-reactions seen in actively sensitized guinea pigs, it is evident that treatment of guinea pig skin with various simple substances, for sensitizing and for testing, will lead to the formation of corresponding conjugates all having in common a moiety, probably protein, with cavy specificity. Experience with artificial conjugated antigens (19), however, has shown the wisdom of selecting different protein components for the antigens to be used for testing and those used for immunizing in order to avoid reactions that depend upon structures other than the attached chemical radical. Such for instance would be areas of the protein surface having altered properties because of immediately contiguous sites of coupling, or spatial structures at the points of attachment, including the basal portion, but not necessarily the entirety, of the attached radical, together with the neighboring portions of the protein molecule. Cross-reactions attributed to both these types of configuration rather than to the whole structure of the attached radical, have been described (19: see pp. 158, 159).

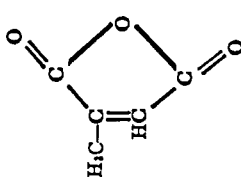
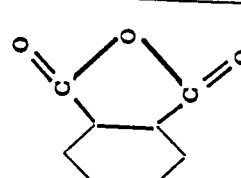
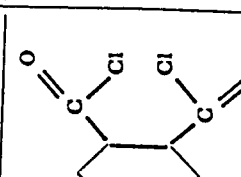
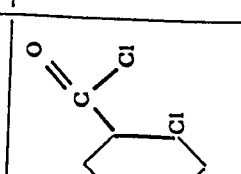
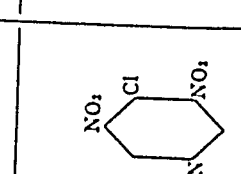
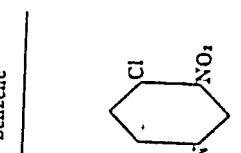
Among our animals, phthalyl chloride in particular led to broad sensitivity, although the homologous reaction was strongest by far. It would seem that some of the cross-reactions may have occurred by reason of configurational structures smaller than the entirety of the simple substance; e.g., reactions in those animals sensitized to phthalyl chloride given by picryl chloride or by picryl casein, the latter at times even leading to anaphylactic shock upon intravenous injection. Citraconic anhydride and *o*-chlorobenzoyl chloride, and their respective conjugates with proteins, have been far enough apart in structure not to give cross-reactions.

The cross-reactions found upon transfer with serum (Table IV) have practically duplicated in scope, and in relative intensity, those observed in the actively sensitized animals. Thus, the sera from phthalyl chloride-treated animals exhibited the broadest range of reactivity, giving cross-reactions even with picryl and dinitrophenyl groupings (*cf.* Table III), likewise when the picryl groupings were attached to casein. The available transfer sera to *o*-chlorobenzoyl chloride exhibited less crossing than did tests on guinea pigs actively sensitized with it,

TABLE IV

## Cross-Reactions with Transfer Sera (Combined Table)

The testing was done by the subcutaneous route, 1 or 2 days after local preparation of the skin. Conjugates were used in amounts of 10 to 100 mg., while the simple substances were employed in 2 mg. test doses except for 1 mg. in the case of citraconic anhydride. The relative strengths of the reactions are shown by the symbols  $\pm$  to  $+++$ .

Test substance	Transfer sera prepared against					
	Citraconic anhydride	Phthalic anhydride	Phthalyl chloride	<i>o</i> -Chlorobenzoyl chloride	Picryl chloride	2:4 Dinitrochlorobenzene
						
Citraconic anhydride	+++	+	$\pm$ to $+\pm$	0	$\pm$	0
Phthalic anhydride	$\pm$	+++	+++	0 <sup>a</sup>	0	0?
Phthalyl chloride	0 to $+\pm$	+++	+++	0 <sup>a</sup>	$\pm$	
Phthalyl guinea pig serum	0 to +++	+++	+++	+++	0	
<i>o</i> -Chlorobenzoyl chloride	0	0	0 <sup>a</sup>	+++	0	
<i>o</i> -Chlorobenzoyl guinea pig serum	$\pm$		(often slow)	+++	0	
Picryl chloride	$\pm$	0	0 to tr.	0	$+\pm$	$+$
Picryl casein	0	0	$+\pm$	0 <sup>a</sup>	+++	
Dinitrophenyl guinea pig serum		0 <sup>a</sup>	0 to $+$	0	$\pm$ to $+$	$++\pm$

<sup>a</sup> The cross-reactions of actively sensitized guinea pigs suggest that with some sera

$\pm$  From cross-tests on actively sensitized guinea pigs

<sup>a</sup> The cross-reactions of actively sensitized guinea pigs suggest that with some sera reactions may yet be found.

$\pm$  From cross-tests on actively sensitized guinea pigs, reaction would not be expected.

despite its partial configurational relationship with substances such as phthalyl chloride and phthalic anhydride. Closer interpretation of such cross-reactions as shown in Table IV require, of course, studies with sera of precisely known antibody content. Skin testing with preformed conjugates would offer a choice of protein moieties and accordingly the opportunity of testing whether reactions are directed only toward the attached chemical radical.

In studying cross-reactivity of sera by passive local sensitization of normal skin, it is to be pointed out that the capacity of a skin area to react may become temporarily exhausted, for

TABLE V

*Passive Local Sensitization of Normal Skin by Sera from Guinea Pigs Sensitized to Cross-Reacting Substances*

Sera injected intracutaneously in amounts of 0.15 cc. Test substances (1 mg.) given subcutaneously in 0.25 cc. corn oil on the 2nd and 3rd days respectively.

Recipient	Test injections		Sera used in preparing skin sites			
	Time	Substance	Anti-citraconic anhydride	Anti-phthalyl chloride	Normal guinea pig serum 3	Normal guinea pig serum 4
No.	hrs.					
1	0	Citraconic anhydride	i = 8' 75 X 62 ++, el., soft, 23'	i = 13' 22 X 22 ±, flat, 43'	Neg.	Neg.
	24*	Phthalyl chloride	Neg.	i = 40' 27 X 27 ++++, el., 75'	Neg.	Neg.
2	0	Citraconic anhydride	i = 7' 75 X 48 ++, el., 27'	i = 12' 23 X 23 +, sl. el., 27'	Neg.	Neg.
	24*	Phthalyl chloride	Neg.	i = 45' 25 X 25 +, sl. el., 90'	Neg.	Neg.
3	0	Phthalyl chloride	i = 21' 29 X 27 +++, m. el., 43'	i = 8' 42 X 35 +++, enorm. 43'	Neg.	Neg.
	24*	Citraconic anhydride	i = 3' 79 X 40 ++, sl. el. 15'	Neg.	Neg.	Neg.

\* All sites appeared normal at 24 hours.

as long as 6 to 8 days, following a reaction; this circumstance may lead one at times to erroneous conclusions when a subsequent stimulation fails to develop another reaction in the area.

#### *Transfer Effects with Common Antigens and the Corresponding Antisera*

The fact that the reactions can be elicited by injection of protein conjugates and not only by the simple compounds themselves naturally suggested a comparison with sera produced with other types of antigenic substances. Actually, reactions of the same type were seen when guinea pig sera prepared against horse serum and against low ragweed were compared directly with a serum obtained after a course of intracutaneous injections of citraconic anhydride (Table VI). No matter what the sequence of injecting the corresponding

test substances was, at each test the homologous prepared site was the only one to respond; further, the responses occurring in the different local areas

TABLE VI

*Passive Local Sensitization of Normal Skin by Various Guinea Pig Sera*

Sera injected intracutaneously 2 days before, in amounts of 0.05 cc. (No. 1) or 0.15 cc. (No. 2). Test substances injected subcutaneously.

Recip- ient	Test injections		Sera used in preparing skin sites			
	Time	Substance	Anti-citraconic anhydride	Anti-ragweed	Anti-horse serum	Normal guinea pig serum
No. 1	0	Horse serum 1 cc. 1:2	Neg.	Neg.	i = 15' 31 × 25 ++, m. cl., 29' 44 × 30 +, m. swol., 51' 44 × 30 ±, v. sl. cl., 120'	Neg.
	2	Ragweed extract 1 cc. "2.5%"	Neg.	i = 8' 22 × 18 +, 13' 25 × 20 ±, cl., 29'	No change	Neg.
	4	Citraconic anhydride 1 mg. in 0.25 cc. oil	i = 4' 70 × 34 ++, cl., gray umbilicate center 17 × 16, 14' 75 × 46 cls., m. cl., 35'			Neg.
2	0	Citraconyl conjugate with guinea pig serum 15 mg. in 1 cc. saline	i = 12' 25 × 25 ++, cl., 25' 23 × 23 al. cls., sl. cl., 180'	Neg.	Neg.	Neg.
	3	Ragweed extract 1 cc. "7%"	No change	i = 15' 25 × 25 +, 42' 27 × 27 ±, 80' tr., 180'	Neg.	Neg.
	6	Horse serum 1 cc. 1:2		No change	i = 23' 30 × 25 +, cl., 48'	Neg.

followed closely a common pattern. One will note that "citraconyl guinea pig serum," a preformed conjugate, elicits the reaction just as does the highly reactive chemical substance itself.

Other types of common antisera produced in guinea pigs and rabbits were investigated in the same manner (4) and upon intracutaneous injection were found to prepare cavy skin for

local reactions. In the case of immune sera from rabbits, as contrasted with sera from guinea pigs, zonal phenomena have been encountered with respect to the relative quantities of antibody and antigen to elicit optimal responses in guinea pig skin. Apart from problems posed by the toxicity of rabbit serum—less than half of the guinea pigs injected with undiluted rabbit antiserum have proved useful in that they have shown only transient, mild erythema with suitable recovery within 2 or 3 days—we have usually found it advisable to prepare the skin with rabbit immune sera diluted 1:5 to 1:30 (*cf.* 4). Both guinea pigs and rabbits, after injections of dead tubercle bacilli in a hydrocarbon vehicle, have produced sera, largely of anticarbohydrate specificity, which give immediate local reactions of the type described above following subcutaneous injection of old tuberculin; this study is still in progress.

### *Passive Sensitization of the Entire Skin*

For preparation of the skin, the local injection of antibody may be replaced, less satisfactorily, by subcutaneous, intraperitoneal, or intravenous injection of larger quantities of the same immune serum, the skin being then generally reactive (and responsive to successive tests over a period of several days). Here also, the reactions are erythematous, edematous, and transient, but they have been less sharp and discrete than in passive local sensitization of the skin. For instance, quantities of the order of 25 cc. given intraperitoneally 1 or 2 days before testing of the skin can be used in place of local injections; *e.g.*, with citraconic anhydride immune serum. In this case, the skin responses can be developed either by scratch testing with citraconic anhydride or by the intracutaneous injection of preformed conjugate, as "citraconyl guinea pig serum."

Transfer experiments of this nature, with antiprotein sera, were conducted previously by Dienes, who injected large amounts of high titered homologous antiserum into normal guinea pigs by the intraperitoneal route and studied the development of the "evanescent type" of skin reaction in response to intracutaneous injection of the corresponding antigen (20; *cf.* 21). Ramsdell (13) likewise on occasion gave antibody intraperitoneally, or subcutaneously, and made a test injection of antigen in the ear or intracutaneously on the abdomen; at the same time, trypan blue was injected into the blood stream in order to facilitate reading of the reactions; her chief experiments are described below. More recently, Fell, Rodney, and Marshall (22) have employed trypan blue similarly in transfer experiments in the rabbit.

### *Transfer Reactions and the Use of Vital Dye*

Ramsdell (13, 17) carried out transfer experiments in guinea pigs and rabbits with immune serum against horse serum prepared in both species, and cleverly employed an intravenous injection of trypan blue immediately prior to the test injection of antigen, so that the area participating in the immediate skin reaction became delineated by local accumulation of dye in consequence of alteration in capillary permeability owing to the antigen-antibody reaction (*cf.* 23). In her experiments, the ears were found to offer the most sensitive test areas, but a few tests were made on the belly skin on the guinea pig. The ear of both the rabbit and the guinea pig could be passively sensitized by local

injection of antibody, and the reaction elicited by the injection of antigen into the same or a contiguous site (13, 17, 24, 25). Other findings are described later; here it may be remarked only that the method was sufficiently sensitive to demonstrate reversed passive reactions, that is, the occurrence of reactions immediately upon injection of antibody into the skin, the antigen having been injected intravenously at a prior time.

Using in place of trypan blue the less diffusible dye pontamine sky blue 6B, freed from salts as described by Parsons and McMaster (26), we made several attempts to employ an intravenous injection of the vital dye in the hope of identifying in the sera of our sensitized guinea pigs antibodies present in lesser concentrations than were detectable visually. It eventuated, however, that sub-erythema reactions were not demonstrable by use of the dye. (More promising were some trials at building up the antibody concentration in a limited area of the skin, by depositing in it on each of 3 successive days three closely adjacent blebs of undiluted antiserum, and testing on the 4th day.)

In these experiments, various dilutions of a few types of guinea pig antisera in saline or in normal guinea pig serum were used for preparing skin sites, and some recipient animals were tested with the specific allergenic material alone, others with this and dye in addition.<sup>6</sup> With preparatory injection of antibody in the skin of the back, it eventuated that blueness developed in the various sites with intensities (and with speeds) varying with the preparatory concentration of immune serum, and the manner of development of the reactions was portrayed delicately, but the dye would accumulate only in areas of antibody concentrations capable of showing visible reactions without the dyestuff. A preliminary, direct comparison of the ear and the skin of the back with use of vital dye yielded essentially equivalent effects, and while our presumptive hope might have been realized by more extended tests on the ear skin (*cf.* 17), these were not pursued, for the latter site was not suitable for the bulk of the work, such as comparative testing of several sera at one time, and the dye method itself was obviously inapplicable when successive tests were to be made on one recipient.

### *Antibody Concentrations; Quantitative Relations in Eliciting the Reaction*

As for the concentration of antibody in guinea pig sera which effect local cutaneous transfer, reactions in the case of the better sera were obtained in sites prepared with 1:8 to 1:30 dilutions in saline or normal serum. Antibodies were at times demonstrable in ring tests with soluble protein conjugates (as also in artificial agglutination tests using as agglutinin soluble protein conjugates adsorbed to collodion particles<sup>7</sup>), but in other cases have not been surely

<sup>6</sup> The technique, as adapted from unpublished data which Dr. Philip McMaster kindly made available, is as follows: 1.5 mg. pontamine sky blue 6B in 1 cc. saline was injected intraguineally into a prepared recipient guinea pig of about 300 gm. weight (5 mg. per kilo) at a time when the reaction could be expected to commence within the succeeding 20 to 30 minutes; accordingly, in some cases the subcutaneous test injection preceded rather than followed the dye injection. Injection of the dye should, however, be made prior to the development of a visible erythematous reaction, for local edema hinders accumulation of the dye.

<sup>7</sup> The collodion particles were kindly supplied by Dr. Jules Freund (27).



detectable except by actual trial in skin transfer experiments; which therefore has been the method of choice (*cf.* 28, 25).

Anaphylactic antibodies were present in far higher concentration than in the sera studied previously (1), where 1 cc. amounts of the best antisera to picryl chloride, and usually 3 cc. of the common antisera, were necessary to give, upon injection into a normal female guinea pig, demonstrable sensitization of uterine horns in the Schultz-Dale test; and transfer was not then feasible with sera from animals treated with 2:4 dinitrochlorobenzene. The higher concentrations of antibody now developed to both of these substances have been, we may conclude, the result of successive restimulations with the allergens over a longer period and of a method allowing readily a more careful selection of sera. In particular, it was now found that upon transfer of antiserum developed to 2:4 dinitrochlorobenzene (4 cc.) the normal recipient animal developed definite anaphylactic symptoms when injected intravenously on the next day with 20 mg. dinitrophenyl guinea pig serum. With the other substances studied in the

TABLE VII

*Passive Anaphylactic Transfer with an Antiserum Obtained after  
Injections of Citraconic Anhydride*

Serum injected by intraperitoneal route, diluted to a total volume of 3 cc., 24 hours before the test shown; the recipients weighed 340 gm.

Recipient	Volume of serum injected	Intravenous injection of 5 mg. citraconyl guinea pig serum
No.	cc.	
1	2.0	Typical anaphylaxis. Death 3'
2	1.5	" " " 4'
3	0.5	" " " 4'
4	0.5	Coughs, jerks, severe convulsion; survived
5	0.2	Negative

present paper, the antibody level attained will reflect their greater chemical reactivity, as well as the length of the sensitization period. As seen in Table VII, fatal anaphylactic shock was demonstrable on the day following transfer of 0.5 cc. of one serum developed to citraconic anhydride. The antibody levels may be compared with the findings in the quantitative experiments of Kabat and Boldt (29) on homologous passive transfer with sera from guinea pigs immunized with ovalbumin: with their sera of high antibody content secured by means of the Freund adjuvant technique and supplementary courses of injections as well, it may be calculated that 0.1 to 0.15 cc. was necessary to lead to fatal anaphylaxis. With an anti-horse serum guinea pig serum prepared by us with the same adjuvant method, but less intensively, only 0.2 cc. was necessary to induce a sensitivity that resulted in fatal shock within 5 minutes when 0.1 cc. horse serum was injected intrajugularly 24 hours later.

The effect of varying the amounts of an antiserum and of its corresponding test substance (citraconic anhydride) is shown in Table VIII. As less of the test substance was employed, both the size of the reactions and the degree of edema decreased, and this effect was the more marked the smaller the amount of antibody used for preparing the site. When so little chemical allergen was used that reactivity was not elicited (recipient 4), the site remained as it were poised, and responded to a later adequate test. When, however, the amount of allergen was such as to induce some partial reaction (recipient 3), this amount sufficed to exhaust the reactivity. The same situation held when the experiment was repeated with an anti-horse

serum guinea pig serum and varying amounts of horse serum were used to elicit the reaction. In both cases, the smaller amount of antibody required a greater amount of allergen or protein in order to cause a reaction, a relationship which appears to differ from that obtaining in the transferred Arthus phenomenon in the rabbit (15). Had the concentration of antibody in the serum been considerably higher, as in rabbit immune serum, the highest test dose might well have given only a transient graying of the skin, and complete discharge ("flash" reaction).

TABLE VIII

*Effect of Varying the Amounts of Antiserum and Allergen on the Transfer Reaction*

Skin sites prepared with 0.15 cc. of dilutions in saline of an antiserum developed by courses of injections with citraconic anhydride. One day later, the various amounts of citraconic anhydride were deposited subcutaneously in 0.25 cc. of corn oil. On the day after the recorded reaction, the skin sites again appeared normal. Only one measurement is given when both diameters were alike.

Recipi- ent	Test dose	Skin sites prepared with serum				Time
		0.1 cc.	0.05 cc.	0.025 cc.	0.0125 cc.	
No.						
1	2 mg.	i = 8' 24 +±, m. cl. 27 × 24 ±, " "	i = 8' 21 +, sl. cl. 22 ±, cl.	i = 8' 21 +, sl. cl. 22 ±, cl.	i = 8' 17 +, sl. cl. 18 ±, v. sl. cl.	20' 55'
2	0.5 mg.	i = 18' 17 +±, not el. 20 ±, " "	i = 18' 14 +±, not cl. 18 ±, " "	i = 18' 14 +, not cl. 18 ±, " "	i = 18'? 12 ±, not el. 12 al. cls., " "	30' 70'
3	0.1 mg.	i = 26' 18 ±, (incomplete), not el. 24 al. cls., cl.	i = 26' 11 ±, not cl.	i = 26' 7 ±, not cl.	i = ? 0? V. sl. reac.	33' 70'
4	0.02 mg.	Pos.?	Pos.?	tr.	tr.	55' 150'
3 (re- tested)	1 mg. 1 day later	Sl. diffuse color over 28 × 16, Sl. color	0  Sl. color	0  V. sl. color	0  Prac. 0	15' 55'
4 (re- tested)	1 mg. 1 day later	25 +++ , m. cl. 29 ±, " "	20 +++ , sl. cl. 25 ±, m. cl.	18 +++ , el. 22 al. cls., el. (soft)	13 faintly brown- ish, not el. 18 cls., el. (soft)	20' 55'

*Observations on the Nature of the Antibody*

There is, as described, a relatively high concentration of anaphylactic antibody in the transfer sera. The question whether various antisera of one specificity exhibit a constant relationship between the quantities conferring passive anaphylactic transfer and local cutaneous preparation of the skin was not answered definitely, because of technical obstacles, but no sharp differences were observed (*cf.* 17). This relationship has been questioned in several reports, as (14), and in studies of some human antibodies (24, 25, 30).

In a few experiments with heating of the transfer serum, there was no clear-cut heat lability such as is seen in the case of the human antibodies termed "reagins" with reference to their property of sensitizing normal human skin (*cf.* 31) and as is reported to occur in the sera of cows actively sensitized with extract of ragweed pollen (32). Rather, we observed that with progressive heating the activity of the sera declined gradually. With an antiserum developed by injections of citraconic anhydride, heating at 60°C. for 30 minutes evidently destroyed half or more of its activity, but the serum was still weakly active when the heating was prolonged at 60° to 1 hour or was done at 63° for 30 minutes. With a guinea pig antiserum to horse serum, the activity was reduced by two-thirds upon being kept at 65° for 2 hours, but there was almost no alteration upon heating at 56° for 4 hours, a treatment usually found adequate to "destroy" the reaginic antibodies in human sera. The effectiveness of heat in altering human reaginic sera likewise in part may depend upon concentration of antibody: thus in the experience of Schmidt and Lippard (31) heating at 56°C. had to be prolonged to 7 hours with one human serum out of thirteen, and it was found that this particular serum had the highest concentration of antibody. As far as the guinea pig antisera are concerned, it would seem unwise at present to view the portion first rendered ineffective by heat as belonging in a special "labile" category.

#### DISCUSSION

In the foregoing it is shown that guinea pigs sensitized to some simple chemical compounds have circulating antibodies which are capable, upon transfer, of inducing skin reactivity of the immediate type.<sup>8</sup> After the manner of testing used with human reagins, the reaction can be elicited in the prepared sites either by local application of reactive materials as in the Prausnitz-Küstner test or by injection at a distant point as in the "*Fernauflösung*" reaction of Jadassohn and others (*cf.* 33, 34, 35). The reaction in guinea pig skin consists of erythema, often spreading pseudopodially, and marked edema, all developing in the course of some minutes under proper conditions of test and receding within an hour or so. The reaction undoubtedly is the same as the "evanescent" type studied by Dienes (20, 21) in guinea pigs sensitized to egg white, and it reproduces the "wheal-and-erythema" type of reaction observed by Jacobs *et al.* (6, 8, 9) on animals actively sensitized to anhydrides.

Early reactivity upon contact with the incitant is not commonly encountered in hypersensitiveness to simple chemicals, although such effects have been observed. Very definite reactions occurring after a short time were found in

<sup>8</sup> The experiments here presented deal with the transfer of reactions of the immediate type; studies on the transfer of reactions of the delayed (contact dermatitis) type have been communicated preliminarily (3), in which case the mechanism of transfer involves the transfer of white cells from the sensitized animals rather than of their serum.

guinea pigs by Jacobs (9), who used as sensitizers anhydrides such as citraconic anhydride or, with less effect, an acyl chloride. In human beings immediate reactions have been described with salvarsan (36, 37) and, in a case of exquisite hypersensitivity to formaldehyde, Horsfall experienced reactions within 10 minutes after contact with 1:30,000 formaldehyde solution.<sup>9</sup> Also, in a patient sensitive to phthalic anhydride Kern (16) saw wheal reactions within 20 to 30 minutes after making a scratch test, and in a more extensive study Feinberg and Watrous (38) observed immediate reactions to chloramine-T (sodium *p*-toluenesulfonechloramide) and to halazone (*p*-sulfonedichloramido-benzoic acid) in factory workers who had become sensitized to these compounds. Also, patients giving immediate reactions to sulfathiazole (39) and to sulfadiazine (40, 41) have been studied experimentally. Kern (16) with phthalic anhydride, Feinberg and Watrous (38) with chloramine-T and halazone, Shaffer, Lentz, and McGuire (39) with sulfathiazole, Whittemore and de Gara (40) and Sherman and Cooke (41) with sodium sulfadiazine, and Ensburner (37) with salvarsan—see also Biberstein (42)—have all reported passive transfer of reactions of the immediate type in skin sites prepared with serum from highly selected allergic individuals. Such positive reports are, however, relatively rare in the extensive studies on human allergy, and most observers have failed in their attempts. Consequently for long only minor consideration was given in the literature to affirmative statements.

The contrast between the ease with which transfer can be effected in experimental animals, once the method has been established, and the generally negative transfer experiments in human beings is probably to be explained by the necessity of securing sera with a sufficiently high concentration of antibodies and by working with substances of high chemical reactivity. With regard to the first point, the animals that produced active serum had been subjected to fairly prolonged treatment, like the factory workers mentioned by Kern, and by Feinberg and Watrous, and the potency of the sera varied according to the time at which they were drawn. Secondly, as to the nature of the substances used, potent transfer sera (and sera allowing passive transfer of anaphylaxis (7, 1)) were most readily obtained, and reactions in prepared skin sites took place most quickly with substances of high reactivity, such as acyl chlorides and anhydrides, and in this category would be included both phthalic anhydride (16) and chloramine-T (38), although interpretation of the latter is at the moment puzzling.

But even with the less reactive compounds the presence of transfer antibodies was demonstrable: although the reactions following injection of the simple compounds might be delayed for some hours or even fail to develop, typical effects were seen when the releasing injection was made not with the

<sup>9</sup> Horsfall, F. J., Jr., personal communication.

simple compound but with a protein conjugate thereof. In this manner transfer reactions were effected not only with the sera of guinea pigs treated with picryl chloride but also with guinea pig antisera against the typical human allergen 2:4 dinitrochlorobenzene. Animals being sensitized with the less reactive allergens, therefore, may possess such circulating antibody but may fail to reveal it either by early responses to intracutaneous test or through transfer attempts with their serum, unless there are available readily reactive conjugates or analogues of the substances in question. It may be anticipated, therefore, that the detection of early-type reactivity in human cases likewise will depend on the nature of the chemical allergen or on the use of preformed conjugates for testing.

Since the corresponding protein conjugates served so well to elicit the reactions, antisera to horse serum and to ragweed extract were prepared in guinea pigs for comparison. Such sera gave the same type of reaction, but as in the case of drug hypersensitivity sera it was necessary to continue the immunization of the guinea pigs for 7 to 9 weeks; with the adjuvant technique of Freund (11), however, adequate concentrations of antibody to horse serum were obtained regularly. Rabbit antibody of several specificities (4) was found to sensitize guinea pig skin in the same way; for preparing skin sites it proved best to dilute powerful precipitating sera five- to thirtyfold. The effect of this, according to the values for antibody content given by Kabat and Boldt (29), would be to reduce the antibody concentration down towards the level that is met with in guinea pig immune sera.

Transfer of antiprotein sera from the rabbit and guinea pig into guinea pigs for the study of cutaneous reactivity had been made by Dienes (20, 21) and by Ramsdell (13, 17), as described above. The method selected by Dienes—intraperitoneal introduction of large amounts of antiserum—allowed careful observation of the "evanescent" type of reaction and served well for his special studies but in our hands has not yielded nearly so sharp reactions as has the local preparation of the skin. Ramsdell, working with sera which probably contained lesser concentrations of antibody than we have selected for use, but employing an intravenous injection of trypan blue to delineate local cutaneous reactivity and selecting a sensitive site (the ear skin), likewise injected antibody intraperitoneally, or subcutaneously, and tested the skin; she could demonstrate even reversed passive reactions. Later, the ear skin of normal guinea pigs was sensitized locally by injection of antibody, and the reactions were elicited a day later by injecting antigen into the prepared site and trypan blue intravenously or giving intravenously a mixture of antigen and trypan blue (17). By the latter procedure, Tuft and Ramsdell (24, 25) could detect antibodies in human sera from a case of horse-serum sickness,<sup>10</sup> and at times

<sup>10</sup> Karelitz and Glorig (30) obtained reactions with such sera in the Prausnitz-Küstner test in human skin.

antibody in the serum of asthmatic patients (43). Ramsdell, then, was the first to reproduce in guinea pig skin reactions "giving a counterpart in the experimental animal of the Prausnitz-Küstner reaction in man." The technique of the present paper (4) has since been used by Kulka and Hirsch (14) for testing rabbit antibody prepared against ragweed extract, and most recently Parventjev, Goodline, and Virion (44) developed reactions in guinea pig skin sites prepared with rabbit antiserum to a bacterial nucleoprotein (from *Hemophilus pertussis*), by testing the skin sites 1 day later with local injections of the antigen.

It would not seem improbable that the antibodies concerned in the cutaneous transfer in the guinea pig are the same as or are closely related to anaphylactic antibodies. This question has not been worked out on a sufficiently large scale. Several of the sera here used were tested for passive anaphylaxis, quantities as small as 0.25 to 0.5 cc. sufficing to induce the anaphylactic state, demonstrable by fatal shock upon intravenous injection of protein conjugates. When sera were heated, the skin-sensitizing activity was seen to fall off gradually as heating was prolonged or the temperature was increased, without evidence of the clear-cut heat lability said to be characteristic of human reagins. After being maintained at 60° C. for 30 minutes, a procedure deleterious to reagins, a serum showed the loss of half or more of its skin-sensitizing ability, but was not entirely inactivated in the same period of time at 63° C. Whatever the nature of the antibody, it has proved possible with guinea pig antiserum against phthalic anhydride to reproduce in the guinea pig the transfer effects observed by Kern (16) with human serum from a patient sensitive to phthalic anhydride.

The relative persistence of the transfer antibodies in guinea pig skin—6 days to 3 weeks or more—also deserves mention, for it approaches the behavior of human reagins deposited in the skin of normal human subjects, and stands in apparent contrast to observations on the shorter period of retention of rabbit agglutinins and rabbit precipitins after injection of these into normal rabbit skin (45). There are some other points of difference, whether or not referable chiefly to different antibody levels attainable in the two species. For instance, in the rabbit there appears to be essentially no incubation period before the prepared site is capable of reaction (13), whereas there is an incubation period in the guinea pig of 7 to 8 hours. Again, the rabbit shows a transferred Arthus reaction, absolutely or in degree unlike the guinea pig. Species differences obviously come into play, and so they may in other instances, such as in comparisons of human and guinea pig antibodies.

It is evident that the production of transfer antibodies, although secured by use of a sensitizing procedure (repeated injections into the skin), is in part at least independent of the concomitant development of delayed-type skin reactivity, for these antibodies were found also in guinea pigs that had been injected intraperitoneally with picrylated stromata of guinea pig erythrocytes

but did not exhibit any contact-type skin sensitivity. In the same way, the immune sera recently reported by Gell, Harington, and Rivers (46) as having been developed in rabbits by courses of injections with interesting new sensitizing substances may be expected to behave like our guinea pig antisera in transfer experiments.

The sera which are useful are those with a sufficiently high level of antibody;<sup>11</sup> the output of antibody fluctuates and may well fall or remain below detectable levels. Hence a low or undetectable concentration of circulating antibody need not reveal a correspondingly low state of sensitivity of the tissues of the actively sensitized animals (for instance, with respect to the anaphylactic state or skin reactivity), a correlation which has often been sought or inferred to exist (e.g., 14, 47).

Turning to the subject of specificity it may be said that the essentially specific nature of the reactions was clearly demonstrated in several cases, for instance in the reactions of citraconic anhydride and *o*-chlorobenzoyl chloride (Table I) and of the latter substance and picryl chloride (Table II), where no overlapping was observed. In other instances in which cross-reactions were encountered, e.g., phthalyl chloride-citraconic anhydride (Table V, cf. Table IV), specificity was clearly demonstrable on a second injection made at a later time, since a prior testing with the heterologous substance left a substantial residual reactivity for the homologous compound, which is evidently akin to partial absorption *in vitro*. The strongest cross-reactivity met with, namely between citraconic anhydride and phthalic anhydride or phthalyl chloride, is plausibly referable to chemical similarity of the substances, all being derivatives of dibasic acids with adjacent carboxyl groups. There were, besides, cross reactions between compounds without obvious relationship in constitution, which occurred as well in the actively sensitized guinea pigs; *i.e.*, a response to picryl chloride or to picryl casein being seen in animals sensitized to phthalyl chloride and in some measure in those sensitized to *o*-chlorobenzoyl chloride, and to *o*-chlorobenzoyl chloride in animals sensitized with phthalyl chloride. Here the explanation may be that the reactions were due more to proteins altered in a similar manner than to the radicals attached to these.

In our hands, reverse transfer was not fruitful, namely securing a reaction by injecting allergen or conjugate some hours to a day prior to the local or intravenous injection of antiserum. Although positive effects had been obtained by Ramsdell (13) in guinea pigs following intravenous injection of the antigen, with trypan blue to outline the reactions, we did not find the unmodified procedure at all promising, despite the fact that there is much to recommend it for some human (Voss (48, 49), Karelitz and Glorig (30)) and animal (50) experiments. In one experiment patterned after Voss, the best effect observed

<sup>11</sup> This is well expressed in the individual differences observed among sera from cases of chloramine-T sensitivity by Feinberg and Watrous (38: Table III).

was a colorless swelling over 28 mm., 3 hours after 1.5 cc. anti-horse serum guinea pig serum had been given intravenously, in a site injected the day before with 0.1 cc. undiluted horse serum; another site injected with 0.02 cc. horse serum remained essentially unaffected.

Following a transfer reaction, a temporary local exhaustion of the capacity of the skin to react was noted, lasting perhaps 6 to 8 days. It would seem that this exhaustion of a skin area may be a secondary phenomenon, not reflecting failure of the antigen-antibody system to react. It may well be a vivid example of the mechanism underlying so called "non-specific antianaphylaxis," *e.g.*, the occasional failure of tissues, as segments of small intestine, from guinea pigs sensitized to a multiplicity of antigens, to respond in the Schultz-Dale test to more than the first antigen tested (51-54). Its counterpart was probably encountered by Ramsdell (13) in the ear of a rabbit actively sensitized to horse serum, in which after a primary reaction to a 1:10,000 dilution of antigen the ear remained insensitive to reinjection for 2 days. It calls to mind, with regard to the "wandering" of urticarial reactions on a patient, the frequently encountered "skipping" of skin areas that have recently reacted (*cf.* 55). Other instances, in man, of temporary local refractoriness following whealing in normal skin are known and have been studied, both after specific Prausnitz-Küstner tests (56) and after transcutaneous introduction, by electrophoresis, of certain drugs, pilocarpine and eserine in particular (55); such effects and those to be seen in guinea pig skin following specific reaction would appear to be closely alike. Pertinent theoretical conclusions have been presented by Alexander, Elliott, and Kirchner (55).

#### SUMMARY

Evidence is presented to show that guinea pigs actively sensitized to simple chemical compounds form serum antibodies capable of sensitizing the skin of normal guinea pigs. Skin sites prepared as for the Prausnitz-Küstner test develop immediate-type ("evanescent") reactions with erythema and edema, upon subsequent injection of the corresponding simple compounds or protein conjugates thereof, and give effects resembling transferred reaginic reactions as seen in human beings. The antibodies were obtainable after sensitization by acyl chlorides, acid anhydrides, and also substances of lesser reactivity, picryl chloride and 2:4 dinitrochlorobenzene, which are human allergens. Observations are reported on the specificity of the antibodies and on various details of the reaction.

Like effects result when antiprotein immune sera and their corresponding antigens are employed for the test, making it highly probable that the antibodies secured after sensitization to drugs result from immunization by conjugates formed *in vivo*.

The sera obtained after sensitization with simple chemical compounds readily



confer passive anaphylaxis, and their capacity for sensitizing the skin declines gradually with progressive heating.

It was observed that following a reaction of substantial degree in guinea pig skin the area involved does not fully recover for some days its capacity to react, the effect being a manifestation, it would seem, of what has been termed "non-specific antianaphylaxis."

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